## REMARKS

This Response is filed in connection with the Office Action mailed May 15, 2007. Claims 31, 34 to 36, 38, 40, 43, 47, 49, 51, 54, 55, 71 to 73, 76, 78, 80, 82, 85 to 88 and 114 to 120 are pending. Claims 54, 85, 128, 139 and 144 to 155 have been cancelled herein without prejudice. Applicants maintain the right to prosecute the cancelled claims in any related application claiming the benefit of priority of the subject application. Accordingly, upon entry of the Response, claims 31, 34 to 36, 38, 40, 43, 47, 49, 51, 55, 71 to 73, 76, 78, 80, 82, 86 to 88, 114, 115, 118, 119 and 121 to 127, 129 to 138 and 140 to 143 are under consideration.

Applicants wish to thank the Examiner for the Interview held May 11, 2007, at which time all grounds for rejection of record were discussed. Applicants believe that the amended claims are consistent with the discussion and address all grounds for rejection of record.

*Regarding the Claim Amendments*

The amendments to the claims are supported throughout the specification or were made to address informalities. In particular, the amendment to claims 31 and 121 to insert the term "diabetes" following treating or treatment was made to more clearly indicate the disorder treated. The amendment to claims 31, 71, 121 and 133 to delete reference to "stem cells, or multipotent progenitor cells that differentiate into K cells" and "stem cells, or multipotent progenitor cells that differentiate into gut endocrine cells" was made to clarify the cell type that produces insulin. In this regard, stem cells and multipotent progenitor cells can be transformed according to the claimed methods, and K cells produce insulin. The amendment to claims 47, 48, 78, 79, 118, 119, 126, 127, 136, 137 and 143 to delete "transformed" was made to provide proper antecedent basis for the cells recited in the claims. The amendment to claims 47, 48, 77, 78, 126, 127, 136 and 137 to recite "are" instead of "is" was made to correct a grammatical error. The amendment to claims 121, 126, 127, 132 136 and 137 to recite "K" cells was made to more clearly indicate which cells produce insulin. The amendment to claim 138 was made to define the gut endocrine cell types with greater particularity. These amendments are also supported throughout the specification, as described in the record. Thus, as the

claim amendments were made to address informalities and are supported by the specification, no new matter has been added and, entry thereof is respectfully requested.

I. OBJECTION TO CLAIMS

Various claims stand objected to due to language informalities. Applicants have amended the claims obviating the objections. In terms of what appears to be a request by the Patent Office to amend claims 31, 71, 121 and 133 to insert "the" before "stomach" and "small intestine," Applicants respectfully request that the objection be withdrawn since if Applicant complied with the request the claim amendment could subsequently raise issues under 35 U.S.C. §112, second paragraph, as indefinite due to a lack of antecedent basis. Since the skilled artisan would know what is intended by claims 31, 71, 121 and 133, Applicants submit that less than perfect English is preferable to a possible holding that the claims are invalid under 35 U.S.C. §112, second paragraph, for lack of antecedent basis.

II. REJECTION UNDER 35 U.S.C. §112, SECOND PARAGRAPH

The rejection of claims 54, 85, 121, 128 and 139 under 35 U.S.C. §112, second paragraph, as indefinite, is respectfully traversed. Allegedly, the metes and bounds of certain terms are unclear. [Office Action, pages 3-6]

Claims 54, 85, 121, 128 and 139 are clear and definite prior to entry of the amendments set forth above. Nevertheless, solely in order to further prosecution of the application and without acquiescing to the propriety of the rejection, claims 54, 85, 128 and 139 have been cancelled herein without prejudice and therefore, the rejection as to these claims is moot. Claim 121 has been amended as suggested. In view of the foregoing, claim 121 is clear and definite and Applicants respectfully request that the rejection under 35 U.S.C. §112, second paragraph, be withdrawn.

III. REJECTIONS UNDER 35 U.S.C. §112, FIRST PARAGRAPH, ENABLEMENT

The rejection of claims 31, 34 to 36, 38, 40, 43, 47, 49, 51, 54, 55, 71 to 73, 76, 78, 80, 82, 85 to 88, 114, 115, 118, 119 and 121 to 143 under 35 U.S.C. §112, first

paragraph, as allegedly lacking enablement is respectfully traversed. The grounds for rejection are as set forth in the Office Action, pages 9-27.

Claims 31, 34 to 36, 38, 40, 43, 47, 49, 51, 54, 55, 71 to 73, 76, 78, 80, 82, 85 to 88, 114, 115, 118, 119 and 121 to 143 are adequately enabled. Nevertheless, solely in order to further prosecution of the application and without acquiescing to the propriety of the rejection, claims 54, 85, 128 and 139 have been cancelled herein without prejudice rendering the rejection of these claims moot, and the remaining claims have been amended as set forth above.

The grounds for rejection will therefore be addressed with respect to the amended claims upon entry of this Response. Applicants submit that the amended claims address all rejections of record discussed during the Interview on May 11, 2007.

In particular, claims 31 and 121 have been amended to recite that the insulin producing cells are K cells. Claim 133 has been amended to recite that the leptin producing cells comprise gut endocrine cells.

In terms of chromogranin A promoter activity, Applicants respectfully point out that chromogranin A promoter is reported to be expressed by many different gut endocrine cells. Previously submitted Exhibit 2, a publication by Rindi *et al.* (Ann. N.Y. Acad. Sci. 1014:1 (2004), at page 5, Table 1, column 4 illustrates that chromogranin A (CgA) was detected in all gut endocrine cell types. Consequently, as chromogranin A is broadly expressed in gut endocrine cells, chromogranin A promoter is broadly active in gut endocrine cells. Accordingly, the claimed methods are adequately enabled for chromogranin A promoter in gut endocrine cells, including the cells recited in claim 138.

In view of the foregoing, the skilled artisan could practice the claims without undue experimentation. As such, the claims are adequately enabled and Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph, be withdrawn.

## IV. REJECTIONS UNDER 35 U.S.C. §103(a)

The rejection of claims 31, 34, 35, 38, 40, 43, 47, 49, 51, 54, 55, 87, 114 and 118 under 35 U.S.C. §103(a) as allegedly unpatentable over During *et al.* (U.S. Patent No.

6,503,887) in view of Boylan *et al.* (J. Biol. Chem. 272:17438 (1997)) is respectfully traversed. The grounds for rejection are set forth in the Office Action at pages 13-15.

Claim 54 has been cancelled herein without prejudice. The rejection is therefore moot as to this claim.

Applicants first wish to clarify what appear to be inadvertent misstatements in the Office Action with respect to the cited art. For example, at page 14, fifth paragraph of the Office Action, it is stated that Boylan *et al.* "describes the GIP promoter conferring specific expression in, *inter alia*, K cells, as well as functional subsequences thereof," it is respectfully pointed out that STC-1 cells are a mixed population of cells derived from a mouse neuroendocrine tumor (abstract). Consequently, because STC-1 cells are a mixed population of many different cell types and any number of cell types in the STC-1 cell population could be expressing GIP, it is not accurate to say that Boylan *et al.* describe the GIP promoter conferring *specific* expression in K cells.

An additional example appears at page 15, first paragraph of the Office Action, where it is stated with respect to During *et al.* that "GIP was known to be responsive to, inter alia, glucose, and *the K cells secrete insulin in response to glucose*." (*emphasis added*) To the contrary, During *et al.* fail to show that K cells are capable of secreting insulin at all, let alone in response to glucose or another of the recited nutrients, or that such regulated insulin secretion would provide a reduction in blood glucose. At most, During *et al.* and Boylan *et al.* report that K cells express GIP, and not insulin- insulin is produced by pancreas, "into the portal circulation" (see During *et al.* column 7, lines 35-44; and Boylan *et al.* abstract).

Turning to the grounds for the rejection, in terms of K cells secreting mature insulin, neither During *et al.* nor Boylan *et al.* provide evidence that it was possible that a non-pancreatic cell, such as gastrointestinal K cells, could correctly process and secrete mature insulin to achieve proper regulation of insulin release. In this regard, insulin is naturally secreted by beta cells of the pancreas islets of Langerhans. The beta cells express enzymes, endoproteases PC2 and PC1/3, which are believed to convert pro-insulin to insulin. Carboxypeptidase E then removes two pairs of dibasic residues to produce mature insulin. However, neither During *et al.* nor Boylan *et al.* teach or suggest that gastrointestinal K cells could produce mature insulin. Absent such evidence, it is at

best speculation that gastrointestinal K cells would have enzymes needed to produce mature insulin.

In support of Applicants' position that it is mere speculation that K cells could produce mature insulin, it was known in the art at the time of the invention that secreted proteins may not be processed properly or may be processed differently when expressed in different cells or tissues. For example, mature insulin was reported to be correctly processed only if expressed in certain specialized cells (Exhibit A, Groskreutz *et al.*, J. Biol. Chem. 269:6241 (1994)), see abstract), and proinsulin expressed in liver is not completely processed (Exhibit B, Koldoka *et al.*, Proc. Natl. Acad. Sci. USA 92:3293 (1995)). Exhibits A and B therefore indicate that one skilled in the art would not have reasonably expected insulin to be processed correctly when expressed in a non-pancreatic cell type, such as a gastrointestinal K cell.

A second example is proglucagon, which is processed completely differently when expressed in pancreas as compared to intestine. In pancreatic islet cells proglucagon is cleaved by PC2 to produce glucagon. However, expression of proglucagon in intestinal endocrine cells produces GLP-1, and not glucagon (Exhibit C, Mojsov *et al.*, J. Biol. Chem. 261:11880 (1986)), see abstract). Exhibit C therefore indicates that one skilled in the art would not have reasonably expected that insulin would be processed in gastrointestinal K cells.

In terms of GIP promoter, as discussed above the STC-1 cells employed by Boylan *et al.* are a mixed population of many different cell types any of which could be expressing GIP and therefore Boylan *et al.* fail to provide evidence that GIP promoter activity would be *specific* for K cells. In view of the foregoing, the skilled artisan would not have viewed Boylan *et al.*'s data obtained in STC-1 cells as indicating GIP promoter specific expression in K cells.

Furthermore, STC-1 cells are a mixed population of cell types, including cells that secrete proteins constitutively, and as discussed above processing of insulin varies depending upon the cell types in which insulin is expressed. In this regard, there are no data in During *et al.* and Boylan *et al.* to indicate that the STC-1 cell population reflects gastrointestinal K cell function *in vivo* in terms of production of mature insulin or secretion of processed mature insulin in a regulated fashion *i.e.*, in response to glucose or

any of the other recited nutrients. Thus, the skilled artisan would have had no idea if STC-1 cells would have fairly predicted *in vivo* gastrointestinal K cell function for production of mature insulin or secretion of insulin in response to glucose or any of the other recited nutrients. Consequently, the skilled artisan, in view of the STC-1 data in Boylan *et al.*, would not have had any reasonable expectation of success that gastrointestinal K cells could properly process and secrete insulin- which is normally produced in pancreas islets- *in vivo*.

In sum, in view of the fact that one skilled in the art at the time of the invention would have known that insulin is not properly processed when expressed in non-pancreatic cells and tissues, and that processing of other peptide hormones, such as proglucagon is altered when expressed in pancreatic islets versus gastrointestinal endocrine cells, the skilled artisan would not have reasonably expected at the time of the invention that insulin, which is normally produced in pancreatic islets, would when expressed in a completely different cell type *in vivo*, namely gastrointestinal K cells, be properly processed to mature insulin. Consequently, there would not have been a reasonable expectation of success at the time of the invention. Further in view of the fact that at the time of the invention one skilled in the art would not have considered Boylan *et al.*'s data obtained in STC-1 cells to accurately representat gastrointestinal K cell function *in vivo* in terms of 1) expression of insulin; 2) secretion of processed insulin; 3) secretion of insulin in response to glucose or the other recited nutrients; and 4) secretion of insulin at levels effective to decrease blood glucose *in vivo*, there would not have been a reasonable expectation of success at the time of the invention. In view of the absence of a reasonable expectation of success there would also not have been a motivation for one skilled in the art to combine Boylan *et al.* with During *et al.*

Finally, it is noted that the Examiner has stated on the record that "insulin was only reasonably predicted to be properly processed and secreted from K cells, and therefore, *simply producing any of the other cells types would not have been reasonably predicted by the Artisan to produce any therapeutic effect in diabetes*." (*emphasis added*, Office Action, page 12, third paragraph) This statement was clearly made in reference to the teaching of Applicants' patent specification, and not in view of During *et al.* and Boylan *et al.* since as discussed above the art of record lacks any evidence to indicate that

insulin could be properly processed and secreted by K cells. In view of this statement, it would not be consistent to assert that During *et al.* and Boylan *et al.* would have provided the skilled artisan with a reasonable expectation of success at the time of the invention.

In sum, During *et al.* (U.S. Patent No. 6,503,887) and Boylan *et al.* (J. Biol. Chem. 272:17438 (1997)) fail to provide the skilled artisan with a reasonable expectation of success or motivation at the time of the invention to produce claims 31, 34, 35, 38, 40, 43, 47, 49, 51, 54, 55, 87, 114, 116 and 118. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be withdrawn.

The rejection of claims 31 and 36 under 35 U.S.C. §103(a) as allegedly unpatentable over During *et al.* (U.S. Patent No. 6,503,887) and Boylan *et al.* (J. Biol. Chem. 272:17438 (1997)) and further in view of Nathan *et al.* (Annals Internal Med. 120:440 (1999)) is respectfully traversed. The grounds for rejection are set forth in the Office Action at pages 15-16.

As discussed above, neither During *et al.* nor Boylan *et al.* provide evidence that K cells correctly process and secrete mature insulin to achieve proper regulation of insulin release from nonpancreatic cells. Absent such evidence, it is at best speculation that K cells would have the enzymes needed to produce mature insulin or that insulin secretion by K cells would be regulated so as to provide a reduction in blood glucose *in vivo*.

As also discussed above, it was known in the art at the time of the invention that insulin is not completely processed when expressed in liver. Proglucagon expressed in the islet cells is cleaved by PC2 to produce glucagon, whereas expression of proglucagon in intestinal endocrine cells produces GLP-1, which was also known in the art at the time of the invention. Thus, one skilled in the art at the time of the invention would not have reasonably expected that insulin would, when expressed in a non-pancreatic gastrointestinal endocrine cell, namely gastrointestinal K cells, be correctly processed, let alone secreted *in vivo* by gastrointestinal K cells in response to glucose or the other recited nutrients at levels effective to decrease blood glucose *in vivo*. Furthermore, the skilled artisan would not have viewed Boylan *et al.*'s STC-1 cells to accurately represent gastrointestinal K cell function *in vivo* in terms of insulin expression, secretion of

processed insulin, in response to glucose or the other recited nutrients, and at levels effective to decrease blood glucose *in vivo*. Consequently, there would not have been a reasonable expectation of success at the time of the invention.

In view of the foregoing, there would not have been a reasonable expectation of success at the time of the invention. In view of the absence of a reasonable expectation of success there would not have been a motivation for one skilled in the art to produce claims 31 and 36.

Nathan *et al.* fail to correct the deficiencies of During *et al.* and Boylan *et al.* In this regard, there is no evidence in Nathan *et al.* that K cells would correctly process and secrete mature insulin to achieve proper regulation of insulin release from nonpancreatic cells. Absent such evidence, there would not have been a reasonable expectation of success at the time of the invention, nor a motivation for one skilled in the art to produce claims 31 and 36.

In sum, During *et al.* (U.S. Patent No. 6,503,887), Boylan *et al.* (J. Biol. Chem. 272:17438 (1997)) and Nathan *et al.* (Annals Internal Med. 120:440 (1999)) fail to provide the skilled artisan with a reasonable expectation of success or motivation at the time of the invention to produce claims 31 and 36. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be withdrawn.

## CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that claims 31, 34 to 36, 38, 40, 43, 47, 49, 51, 55, 71 to 73, 76, 78, 80, 82, 86 to 88, 114, 115, 118, 119 and 121 to 127, 129 to 138 and 140 to 143 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

The Examiner is invited to contact the undersigned to arrange an Interview if any of the issues raised in the Office Action remain upon entry of this response. Applicant's representative can be reached at (858) 509-4065.

Please charge any fees associated with the submission of this paper to Deposit Account Number 03-3975. The Commissioner for Patents is also authorized to credit any over payments to the above-referenced Deposit Account.

Respectfully submitted,

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# Genetically Engineered Proinsulin Constitutively Processed and Secreted as Mature, Active Insulin*

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**The conversion of human proinsulin to insulin occurs only in specialized cells which contain the appropriate processing enzymes. To allow proinsulin processing to occur in a wide variety of cell types, we engineered human proinsulin to be cleaved in the constitutive secretory pathway. Using site-directed mutagenesis, we have introduced furin consensus cleavage sequences (Arg-X-Lys-Arg) into the human proinsulin cDNA. These mutations allowed for efficient proteolytic maturation of human proinsulin to insulin within cells containing only a constitutive pathway of secretion. Additionally, a naturally occurring mutation (histidine B10 to aspartic acid) yields a form of human insulin which accumulates 10- to over 100-fold more mature insulin when compared to the mutants lacking this change. Engineering furin-specific cleavage sites into each junction of the human proinsulin cDNA results in the secretion of peptides that display the expected molecular weights for the A and B chains of insulin. The accumulation of mature, processed, human insulin was measured in the supernatants by radioimmunoassay, and the bioactivity of this molecule was measured by its ability to stimulate autophosphorylation of the insulin receptor. Our results suggest that any cell type might be engineered to produce mature, active, and stable insulin in the constitutive pathway of secretion.**

Proteins are processed and secreted by one of two means in eukaryotic cells: the constitutive or the regulated pathway (1). Insulin is normally processed into the mature, active A, and B chain complex (2) in the secretory vesicles of pancreatic β-cells which contain the requisite processing enzymes (3–6). During processing, the C peptide, which resides between the B and A peptides in proinsulin, is excised by enzymes that make two separate cleavages: one at the B-C junction (Arg-Arg dibasic site), and one at the C-A junction (Lys-Arg dibasic site) (2). In cells with only the constitutive pathway, there are no specialized, regulated secretory vesicles nor are the specialized proinsulin processing enzymes present.

Recently, rat proinsulin II has been shown to be partially cleaved within cells which contain only the constitutive pathway of secretion (6–8). Cleavage occurs at the C-A junction which in the rat protein is the tetrabasic sequence, Arg-*X*-(Lys/Arg)-Arg (7); most likely, cleavage is due to the endogenous, Golgi-anchored, processing enzyme, furin (9–11) or by PACE4 (12), which are present in the constitutive pathway of most cells (13). Although processing was observed in the rat proinsulins which contain one furin site, processing was still minimal due to the absence of furin cleavage sites at both junctions. Smeekens *et al.* (6) were able to improve processing at the B-C junction of rat proinsulin I (Lys-Ser-Arg-Arg) in their expression system by co-transfection with furin and Yanagita *et al.* (8) were able to increase the efficiency of cleavage of the rat precursor by the addition of a furin-type cleavage site.

In contrast, human proinsulin, which lacks any furin consensus sequence, is not effectively converted to mature insulin in cells which contain only the constitutive pathway of secretion. Our goal in this study is to develop a means by which a wide variety of cell types can produce mature, active human insulin. To create non-endocrine cells with the ability to correctly and efficiently process human proinsulin to mature insulin, we took advantage of the fact that there are endogenous, Golgi-anchored, processing enzymes (9–12) present in the constitutive pathway of most cells (13). Using site-directed mutagenesis, we engineered proinsulin to be a substrate for furin by introducing new cleavage sites at both the B-C and the C-A junctions of human proinsulin. Following transfection, the variant human proinsulin cDNA is processed constitutively into active, mature, human insulin. To assess cleavage, protein sequencing was performed on the final cleavage products, and the bioactivity of the secreted molecules was determined by monitoring autophosphorylation of the insulin receptor. We also studied a naturally occurring variant of proinsulin that is present in a subset of patients with hyperproinsulinemia. This variant replaces the histidine at position 10 in the B chain with an aspartic acid (14). When this mutation is introduced into the engineered-processed proinsulin cDNA, the resulting mutant insulin subsequently accumulated at 10- to 100-fold higher levels. Receptor binding and autophosphorylation assays demonstrate that this insulin variant binds and activates the insulin receptor similarly to native insulin.

## MATERIALS AND METHODS

*Plasmid pRK.proinsulin*—The 330-base pair cDNA for proinsulin was amplified from plasmid pHI3 (15) using the polymerase chain reaction (16). The primers used in the amplification created restriction sites *Hin*dIII and *Xba*I (underlined below) which allowed for cloning into the expression vector pRK7, a derivative of pCIS (17). The sequences of the forward and reverse primers were: 5′-CATAAGCTTACCATGGCCCTGTGGATGCGC-3′ and 5′-CATTCTAGACTAGTTGCAGTAGTTCTCCAG-3′, respectively.

*Mutant Proinsulin Constructions*—Site-directed mutagenesis (18) as modified by Kunkel (19) was used to mutate proinsulin at the B-C and/or C-A junctions producing furin or prohormone convertase recognition sequences. Primer sequences were as follows (new restriction sites are underlined): Site I.KR, 5′-CTCTGCCTCCCGCTTGGTCTTCGGTGTGTAG-3′; Site I.RTKR, 5′-CTCTGCCTCCGCTTGGTCCTGGGTGTGTAG-3′; Site II.RQKR, 5′-CACGCTTCTGCCGGGATCCCTC-3′; $Asp^{B10}$, 5′-GCTTCCACCAGGTCGGATCCGCACAGGTG-3′.

Mutagenesis was performed using the Muta-Gene Phagemid *in vitro* Mutagenesis Kit (Bio-Rad) according to the manufacturer's instruc-

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tions. Mutant colonies were screened and then sequenced through the priming area with the Sequenase Version 2.0 Sequeneing Kit (U. S. Biochemicals) according to the manufacturer's instructions.

*Expression and Labeling of Proinsulin and Proinsulin Mutants*—Plasmids were prepared for transfection with Qiagen columns and transfected into an adenovirus transformed human kidney cell line (HEK-293) (20) using the calcium phosphate precipitation method as previously described (17). This procedure follows the transfection protocol described by Gorman *et al.* (21) with two exceptions: DNA precipitates are left on the cells overnight and a plasmid carrying adenovirus VA RNA genes is co-transfected to enhance translation (22). This method has been shown to yield up to milligram quantities of recombinant protein in a few days (23). Cells were labeled as described by Marriott *et al.* (24), and both the secreted and intracellular products were analyzed for insulin-like proteins. Protein A-Sepharose immunoprecipitation was performed (25) on both media and extract samples using concentrated guinea pig anti-human insulin antibodies (Biomedia). Immunoprecipitation products were resuspended in either 2 × Tris-glycine SDS-polyacrylamide gel electrophoresis sample buffer or 8 M urea plus β-mercaptoethanol heated for 5 min at 100 °C, centrifuged briefly, and electrophoresed on 18% Tris-glycine SDS. This polyacrylamide gel electrophoresis system was ideal in that it allowed for separation of the B and A chains of mature insulin by 3–6 mm. The gels were fixed (10% acetic acid/25% isopropyl alcohol) and prepared for fluorography.

*Quantitation of Insulin Products—Radioimmunoassay*—Radioimmunoassay was performed on conditioned supernatants according to the provided instructions (Equate® RIA INSULIN, Binax, Inc.). As stated by the manufacturer, the sensitivity was 0.08 ng/ml, and the coefficient of variation was 8%.

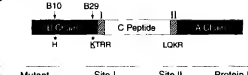
*Insulin Receptor Binding Assay*—NIH 3T3 HIR3.5 cells (26) overexpressing the human insulin receptor (a generous gift from Dr. Ira Goldfine and Dr. Jonathon Whittaker) were incubated in serum-free medium with various concentrations of unlabeled wild type insulin or mutant human insulins and a constant amount of $^{125}$I-insulin (Amersham) for 16 h at 4 °C. Unbound ligand was removed, and cells were washed twice with ice cold medium. The amount of radioactivity bound was determined after solubilizing the cells with 0.1 N NaOH containing SDS (0.1%). Relative binding of wild type insulin and the insulin mutants were fit to a four-parameter nonlinear least squares equation.

*Insulin Receptor Autophosphorylation Assay*—The activity of human proinsulin, bovine insulin, or the various mutant human insulin secretion products was measured by quantitating the increase in tyrosine phosphorylation of the β subunit of the insulin receptor in HEK-293 cells or NIH 3T3 HIR3.5 cells (26). Assays were conducted as described (27) except that cells were stimulated for 10–15 min with bovine insulin (0.01–1000 ng/ml) (Novo Industri A/S) or mutant human insulin from conditioned supernatants. Samples were electrophoresed on an 8% Tris-glycine SDS gel (Novex). After electrophoresis, the proteins were transferred to PVDF,[1] and tyrosine phosphorylation of the β chain of the insulin receptor was detected by probing with mouse anti-phosphotyrosine antibodies followed by alkaline phosphatase-conjugated anti-mouse IgG. Bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega) and quantitated by scanning densitometry as previously described (27).

Additionally, NIH 3T3 HIR3.5 cells were plated to confluency in 60-mm Costar tissue culture dishes and treated as above with the exception that the stimulation reaction was stopped with 500 μl of Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris, pH 8.0). The extracts were immunoprecipitated with anti-human insulin receptor antibodies Ab-1 and Ab-3 (Oncogene Science) or anti-insulin receptor substrate-1 antibodies (UBI). Immunoprecipitation products were electrophoresed and transferred to PVDF membrane, and tyrosine-phosphorylated proteins were detected with anti-phosphotyrosine antibodies as stated above.

## RESULTS

*Human Proinsulin Mutants*—To test whether human proinsulin could be engineered to be processed in the constitutive pathway, a series of human proinsulin mutants were made with altered amino acid sequences at either the B-C (type I) or C-A (type II) cleavage site or at both cleavage sites. These new cleavage sites are based on the putative recognition sites defined for furin (28, 29). Fig. 1 depicts native human proinsulin




| Mutant | Site I | Site II | Protein Product |
|---|---|---|---|
| proins.Ifur.IIfur | RTKR ↓ | RQKR ↓ | LysargArg-insulin |
| proins.IKR.IIfur | KTKR | RQKR ↓ | B-C Intermediate + A chain |
| proins.I-.IIfur | KTRR | RQKR ↓ | B-C Intermediate + A chain |
| proins.Ifur.II- | RTKR ↓ | LQKR | C-A Intermediate + B chain |
| proins.Ifur.IIfur.BcO | RTKR ↓ | RQKR ↓ | LysargArg.HissloAsp-insulin |

FIG. 1. **Schematic of proinsulin and cleavage mutants.** The type I cleavage site (KTRR) is at the B-C junction, and the type II cleavage site (LQKR) is at the C-A junction. The C peptide must be removed during processing to mature insulin. The cleavage mutations made at each cleavage site are shown. *fur* refers to a furin recognition site at either site I or site II. The *arrows* indicate where cleavage occurs in these mutants. The protein products resulting from transfection with the various proinsulin mutants are indicated. Amino acid abbreviations: *R*, Arg; *T*, Thr; *K*, Lys; *L*, Leu; *Q*, Gln.

highlighting the dibasic cleavage sites which separate the C-peptide from the A and B chains. The mutant proinsulin variants are shown below with the added furin-specific cleavage sites. Mutant proins.Ifur.IIfur, contains furin recognition sequences at both site I (B-C junction) and site II (C-A junction). Introducing a furin recognition sequence (Arg-*X*-Lys-Arg) at site I will introduce a point mutation into the final B chain at position B29 (Lys to Arg) resulting in the mutant $Lys^{B29} \rightarrow Arg$ insulin protein product indicated. The variant, proins.IKR.IIfur, tests the requirement of an Arg in the P4 position for cleavage of the type I site. Proins.Ifur.II- and proins.I-.IIfur were each single mutants containing one furin cleavage site. These mutants allowed us to see that efficient cleavage only occurs where there are specific furin recognition sites. The final mutant, proins.Ifur.IIfur.*B10D*, has both furin recognition sequences; however, it also contains a mutation (His to Asp) at position 10 in the B chain which was introduced to enhance stability of the mature insulin product. The protein products resulting from each of the mutants are indicated in the right hand column.

*Expression of Proinsulin and Proinsulin Mutants*—Following transfection of HEK-293 cells with an expression vector carrying the human proinsulin or mutant proinsulin cDNA, cells were labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine followed by immunoprecipitation with anti-insulin antibodies. The $^{35}$S-labeled immunoprecipitated samples were electrophoresed on 18% SDS Tris-glycine gels (Fig. 2). Following transfection with the wild type human proinsulin cDNA, an uncleaved form of proinsulin with a molecular weight of 6,500 can be immunoprecipitated from both the cell medium (Fig. 2, *lane 1*) and the cell extract (Fig. 2, *lane 2*). Thus, in the absence of regulated secretory vesicles which contain the correct processing enzymes, human proinsulin is the predominant secretion product. *Lanes 5–12* illustrate the processed products following transfection with the human proinsulin variants. Only unprocessed human proinsulin is detected in all of the cell extracts (Fig. 2, *lanes 2, 4, 6, 8,* and *10*). No expression differences between each variant were detected as monitored by the levels of proinsulin produced; however, the secreted products differed between the native proinsulin and the mutants. The primary secretion product from cells transfected with native human proinsulin cDNA is unprocessed material (Fig. 2, *lane 1*) although there is a minor amount of processing at the B-C junction which can be detected with longer exposure times (Fig. 2 *lane 3*). However, the products from the proinsulin.Ifur.IIfur

[1] The abbreviation used is: PVDF, polyvinylidene difluoride.

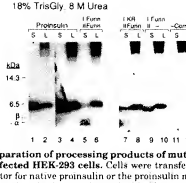



FIG. 2 **Separation of processing products of mutant proinsulin from transfected HEK-293 cells.** Cells were transfected with an expression vector for native proinsulin or the proinsulin mutants outlined in Fig. 1. Transfected cells were labeled with [$^{35}$S]Cys and [$^{35}$S]Met for 4 h. Insulin immunoreactive proteins were immunoprecipitated from both the media and the cell extracts with insulin antibodies. The samples contained 8 M urea and were reduced prior to separation on 18% Tris-glycine gels. *S* indicates cell supernatant, and *L* indicates lysed cell extract. *Lanes 1* and *2* are native proinsulin, an overnight exposure. *Lanes 3* and *4* are native proinsulin, a 4-day exposure. *Lanes 5* and *6* are the proins.Ifur.IIfur mutant, a 4-day exposure. *Lanes 7* and *8* are the proins.IKR.IIfur mutant, a 4-day exposure. *Lanes 9* and *10* are the proins.Ifur.II-mutant, a 4-day exposure. *Lanes 11* and *12* are a nontransfected control. This is a 4-day exposure.

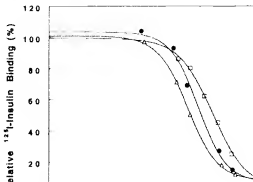



FIG. 3. **Bovine insulin, $Lys^{B29} \rightarrow$ Arg insulin, and $Lys^{B29} \rightarrow$ Arg, $His^{B10} \rightarrow$ Asp insulin have similar, dose-responsive binding affinities.** NIH 3T3 HIR3.5 cells were used to determine the binding affinities of the two insulin variants with wild type insulin standards. *Filled circles* represent binding of the insulin standards, the *open squares* represent the $Lys^{B29} \rightarrow$ Arg insulin binding, and the *open triangles* show $Lys^{B29} \rightarrow$ Arg, $His^{B10} \rightarrow$ Asp insulin binding. The $EC_{50}$ values were 14.1 ± 2.4 ng/ml, 42 ± 17 ng/ml, and 6.4 ± 1.5 ng/ml, respectively.

variant show an increase in the production of mature $Lys^{B29} \rightarrow$ Arg insulin (Fig. 2, *lane 5*). Proins.Ifur.IIfur is processed into products that co-migrate with the A and B chains of insulin as well as processing intermediates. When the furin cleavage site was placed at just one of the two junctions (Fig. 2, *right-hand panel*), only one of the mature chains was detected along with the intermediates. With the furin cleavage sequence at site II (Fig. 2, *lane 7*), the A chain is detected; a slower migrating band consistent with the B-C intermediate is also present. When the furin cleavage site is placed at site I (Fig. 2, *lane 9*), the B chain is detected as is a faster migrating intermediate, most likely the C-A peptide. Intermediate bands disappear when the amount of furin in the cells is increased by co-transfection with an expression plasmid containing a full-length furin cDNA (data not shown). The Lys-Arg dibasic site placed at cleavage site I was resistant to furin cleavage as would be expected due to the lack of an arginine in the P4 position. In the mutant proins.I-.IIfur, the processing products were similar to proins.IKR.IIfur (Fig. 2, *lane 7*) (data not shown).

Sequence analysis of the complete B-chain and of the N-terminal portion of the A-chain confirmed that cleavage was occurring at the furin consensus cleavage sites. Furthermore, removal of the basic residues at positions 31 and 32 of the B chain, leaving the $Thr^{B30}$ as the final amino acid of the B chain, supports the presence of carboxypeptidase in HEK cells (24). Mass spectrometry of the reduced B-chain was consistent with protein sequencing data (data not shown).

*B Chain Insulin Variants*—Previously, it had been shown that changing the histidine residue to an aspartic acid at position 10 of the B peptide increases the stability (30) of mature human insulin. This mutation $His^{B10} \rightarrow$ Asp was made in the cDNA of both native human proinsulin and the proins.Ifur.IIfur mutant. For a direct comparison of the levels of insulin produced by the engineered cDNAs, radioimunnoassays were performed at 24 h following transfection. There was consistently an increase of 10–100-fold in the accumulation of secreted insulin from cells transfected with the $His^{B10} \rightarrow$ Asp mutation when compared to the accumulation levels of the double mutant which lacks the $His^{B10} \rightarrow$ Asp mutation. Depending on the transfection, the concentration of $Lys^{B29} \rightarrow$ Arg insulin assayed in conditioned medium ranged from 8 to 50 ng/ml, whereas the $Lys^{B29} \rightarrow$ Arg, $His^{B10} \rightarrow$ Asp insulin ranged from 300 to 1500 ng/ml.

*Detection of Receptor Binding and Autophosphorylation*—We used an NIH 3T3 cell line overexpressing the human insulin receptor (26) to study the receptor binding properties of native insulin and the insulin mutants used in this study. These assays resulted in relatively similar, dose-dependent binding affinities for wild type insulin and both of the mutants, $Lys^{B29} \rightarrow$ Arg insulin and the $Lys^{B29} \rightarrow$ Arg, $His^{B10} \rightarrow$ Asp (Fig. 3).

The bioactivity of our engineered insulin species was monitored by their ability to stimulate autophosphorylation of the endogenous insulin receptor in HEK-293 cells or the recombinant receptor in NIH 3T3 HIR3.5 cells. Cells were starved in serum-free media and then stimulated with various concentrations of wild type or mutant insulin. After stimulation, phosphorylation of the insulin receptor β-chain was analyzed by Western blot (data not shown). The relative intensities of the 96-kDa tyrosine-phosphorylated bands were quantitated using scanning densitometry of the Western blots, and dose titration curves for both standards and mutants were generated using a four-parameter nonlinear least squares equation (Fig. 4). Serum/insulin-free conditioned medium from mock-transfected cells does not lead to detectable amounts of tyrosine phosphorylation of the 96-kDa protein (data not shown).

Confirmation that the 96-kDa protein monitored in the Western blots (data not shown) is the human insulin receptor β-chain is shown in Fig. 5. Cells were stimulated with insulin and then lysed with buffer containing Nonidet P-40. Proteins were then immunoprecipitated with anti-insulin receptor. The immunoprecipitation products were electrophoresed under reducing conditions, transferred to PVDF membrane, and then probed with anti-phosphotyrosine antibodies. Fig. 5 (*lanes 1–3*) shows that wild type and mutant insulin-stimulated cells (but not unstimulated cells) contain identical tyrosine-phosphoylated proteins of 96 kDa.

In addition to the band at 96 kDa, shown to be the β-chain of the insulin receptor, Western analysis detects a band that migrates with an apparent molecular size of 180 kDa which has a similar increase in tyrosine phosphorylation with increasing concentrations of mutant or native insulin (data not shown). This band is of the same molecular weight as that published for the insulin receptor substrate-1 (IRS-1) (31, 32). Similarly, the IRS-1 immunoprecipitation products contain a protein of 180

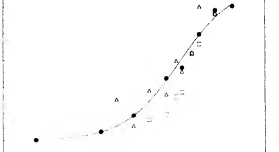



FIG. 4. **Insulin activation of the β subunit of the insulin receptor.** Autophosphorylation of the insulin receptor present on HEK-293 cells was monitored by Western blotting using antiphosphotyrosine antibodies as discussed under "Materials and Methods." Biological activity of bovine insulin and insulin mutants was examined by measuring relative levels of phosphorylation of the β-chain of the insulin receptor on HEK cells with scanning densitometry. The results of three separate experiments are summarized. The *filled circles* represent the wild type standards, the *open squares* represent $Lys^{B28}$ · Arg insulin, and the *open triangles* represent $Lys^{B28}$ · Arg $His^{B10}$ · Asp insulin. The $EC_{50}$ values were 2.5 ± 1.2 ng/ml, 5.3 ± 0.9 ng/ml, and 4.7 ± 3.2 ng/ml, respectively.

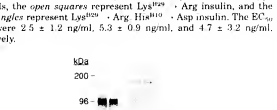



FIG. 5. **Determination of the 96- and 180-kDa tyrosine-phosphorylated proteins as the insulin receptor β-chain and the IRS-1 proteins, respectively.** Anti-insulin receptor (*lanes 1–3*) or anti-IRS-1 (*lanes 4–6*) antibodies were used to immunoprecipitate NIH 3T3 HIR3.5 cell lysates after they had been stimulated for 10 min with wild type or mutant insulin. Immunoprecipitation products were fractionated on 8% Tris-glycine gels, transferred to PVDF, and probed with antiphosphotyrosine antibodies. Cells represented by *lanes 1* and *4* were stimulated with 30 and 300 ng/ml of the mutant $Lys^{B28}$ · Arg, $His^{B10}$ · Asp insulin, respectively. *Lanes 2* and *5* were stimulated with 30 and 300 ng/ml of wild type bovine insulin, respectively, and *lanes 3* and *6* were from unstimulated cells.

kD that is phosphorylated at tyrosine residues in stimulated but not unstimulated conditions (Fig. 5, *lanes 4–6*).

## DISCUSSION

Human proinsulin cannot be processed in cells containing only the constitutive pathway of secretion. This lack of processing is due, in part, to the lack of the appropriate prohormone converting enzymes. Although the enzymes involved in proinsulin processing are present in specialized cells, there are two other members of the mammalian subtilisin enzymes which are widely expressed, furin (9–11) and PACE4 (12).

To allow human proinsulin to be processed by the constitutive pathway, we introduced furin cleavage sites (Arg-*X*-(Lys/Arg)-Arg) at both B-C and C-A junctions of human proinsulin. Our results demonstrate that the human proinsulin cDNA can be modified to allow efficient processing and secretion of active, mature insulin in cells containing only a constitutive pathway of secretion.

The creation of furin recognition sequences at both the B-C and C-A junctions of human proinsulin allowed for correct cleavage by the endogenous enzyme(s) in HEK-293 cells as confirmed by sequencing the entire B chain and N-terminal sequencing of the A chain. Consistent with earlier studies demonstrating carboxypeptidase activity in HEK-293 cells (24), the dibasic amino acids at positions 30 and 31 of the B chain of proinsulin are removed. Our approach results in an amino acid substitution $Lys^{B29}$ · $Arg^{B29}$ in the final insulin product. This conservative substitution in $Lys^{B29}$ · Arg insulin appears to have little effect on either biological activity or insulin receptor binding. Although we have engineered furin cleavage sites into the human proinsulin cDNA and the addition of furin by co-transfection does indeed lead to complete cleavage of these sites, we do not know that it is indeed furin which is responsible for the cleavage within the 293 cells. PACE4 is also expressed in these cells and may also recognize these cleavage sites.

A type of proinsulin variant found in a subset of patients with hyperproinsulinoma contains an Asp rather than a His in position 10 of the B chain. Previously, it has been shown that insulin containing this mutation results in a more stable form of insulin (30), or, alternatively, a more active form of insulin due to higher affinity binding to the insulin receptor (33–35). Since this form of insulin does not form hexamers (36, 37), it may therefore also be more bioavailable. In our system, proins.Ifur.IfurHis*B10*Asp is processed constitutively and yields a 10–100-fold increase in the amount of insulin which is secreted from the HEK-293 cells. This was observed both by radioimmunoassay and in the pulse-chase experiments (data not shown). Both mutant forms of insulin processed constitutively in our experiments, as tested from conditioned media, are similar to wild type insulin in both biological activity and receptor binding. In conclusion, we have engineered human proinsulin so that it is efficiently processed to mature, biologically active, stable insulin via an endogenous protease, perhaps furin, present in the constitutive secretory pathway of many cells.

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# Gene therapy for diabetes mellitus in rats by hepatic expression of insulin

(*in vivo* gene therapy/recombinant retroviral vectors/insulin gene expression/hepatic gene transfer)

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**ABSTRACT** **Type 1 diabetes mellitus is caused by severe insulin deficiency secondary to the autoimmune destruction of pancreatic β cells. Patients need to be controlled by periodic insulin injections to prevent the development of ketoacidosis, which can be fatal. Sustained, low-level expression of the rat insulin 1 gene from the liver of severely diabetic rats was achieved by *in vivo* administration of a recombinant retroviral vector. Ketoacidosis was prevented and the treated animals exhibited normoglycemia during a 24-hr fast, with no evidence of hypoglycemia. Histopathological examination of the liver in the treated animals showed no apparent abnormalities. Thus, the liver is an excellent target organ for ectopic expression of the insulin gene as a potential treatment modality for type 1 diabetes mellitus by gene therapy.**

Type 1 or insulin-dependent diabetes mellitus (IDDM) is caused by the lack of insulin and results from the autoimmune destruction of the insulin-producing β cells of the pancreas (1). In IDDM, a lack of insulin results in wasting, hyperglycemia, and death from ketoacidosis (2–4). The present treatment for IDDM includes careful monitoring of blood glucose levels, multiple injections of insulin, specialized diet, and exercise regimens. With vigorous compliance and intensive diabetes management strategies, patients can expect a 50–70% reduction in the severe microvascular complications of diabetes, but their health and life expectancy are still compromised (5). The present intensive treatment is still suboptimal for glucose control and patient noncompliance limits the universality of such programs. Thus a further treatment modality for IDDM by gene therapy was explored.

Since β cells are destroyed in IDDM, any attempt to reconstitute insulin gene expression must be directed at an ectopic organ. The liver is an obvious choice since it is the main target organ for insulin action and the principal effector organ in maintaining blood glucose homeostasis and ketogenesis. Somatic gene therapy was attempted by hepatic expression of the insulin gene in severely diabetic rats. The goal of this investigation was to test the hypothesis that ketoacidosis in severely diabetic animals can be prevented by sustained, low-level expression of the insulin gene in the liver.

## MATERIALS AND METHODS

**PCR Rescue of a Full-Length Rat Insulin 1 cDNA Clone.** To replace the missing 33 nucleotides from the 5′ end of the rat insulin 1 cDNA (6), the following oligonucleotides were designed: 5′-GCC GCC ACC ATG GCC CTG TGG ATG CGC TTC CTG CCC CTG CTG GCC CTG CTC GTC CTC TGG-3′ and 5′-CC CTC GAG TCA GGT GCA GTA GTT CTC CAG-3′. The PCR product was cloned into the Bluescript vector, and sequencing revealed it was identical to the rat insulin 1 cDNA sequence.

**Construction of a Recombinant Retroviral Vector Encoding the Rat 1 Insulin Gene.** The rat insulin 1 cDNA was cloned into pLNCX (7) and the cytomegalovirus and *neo* genes were deleted. The resulting plasmid, pLX/rINS, encoding the 5′ long terminal repeat (LTR), the rat insulin gene, and the 3′ LTR, was transfected into the retroviral packaging cell line GPAM-12. Individual colonies were isolated and screened on the ability to induce insulin production from rat fibroblast 208F cells.

**Retroviral Transduction of Rat Hepatocytes *in Vivo* and Induction of Diabetes.** *In vivo* hepatic gene delivery using recombinant retroviral vectors was performed as described (8). Briefly, male Lewis rats, 3–4 weeks old, were subjected to a 70% partial hepatectomy. Twenty-four hours later, 4.0 ml of retroviral supernatant were infused into the portal vein. Two weeks after retroviral transduction, diabetes was induced with streptozotocin (at a dose of 250 mg/kg), which was dissolved in 0.1 M citrate buffer (pH 4.5) immediately before intraperitoneal injection.

**Determination of Retroviral Tissue Distribution and Expression.** Three days after the induction of diabetes, three experimental and two control rats were sacrificed, and RNA and DNA was isolated from tissues. Two PCR primers—5′-AGTCCCGTCGTGAAGTGGA, which hybridized to the rat insulin cDNA, and 5′-CCTGACCTTGATCTGAACTTC, which hybridized to the Moloney murine leukemia virus vector—were used to generate a 345-bp product that specifically hybridized to labeled rat insulin cDNA. DNA from various organs as well as pLX/rINS, 10-fold serially diluted in Lewis rat liver genomic DNA, were used as templates for PCR analysis. The intensities of the ethidium bromide-stained bands after gel electrophoresis were compared to estimate the number of LX/rINS proviral sequences per cell in the tissues. For expression studies, mRNA was isolated from total cellular RNA, which was used to generate cDNA. The cDNA was used for PCR using the above primers.

**Serum Chemistry Analyses.** Blood glucose levels were determined using a One Touch blood glucose monitoring system (Lifescan, Mountain View, CA). Serum ketones were determined by spotting serum on an Ames Ketostix reagent strip. For RIA, serum samples were sent to Linco Research Immunoassay Services (St. Charles, MO) for analysis. The insulin RIA utilizes antibodies raised against rat insulin, and the C peptide RIA utilizes antibodies raised against rat C peptide. The glucagon RIA utilizes an antibody raised against human glucagon.

**Histopathological and Immunocytochemical Examination.** For periodic acid/Schiff reagent and hematoxylin/eosin staining, the tissues were fixed in 10% buffered formalin, cut in

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; C:I, C peptide-to-insulin.

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2-μm slices, and stained (9). For fat staining (oil red O), tissues were placed in Shandon cryomatrix, immediately frozen in liquid nitrogen, and stained (9).

## RESULTS

**Construction of a Recombinant Insulin Retroviral Vector.** LX/rINS, a Moloney murine leukemia virus (7)-based retroviral vector, encodes the complete sequence for rat preproinsulin 1 cDNA (6) under the transcriptional control of the viral LTR promoter. Transduction of 208F cells *in vitro* resulted in the production of 564 ng of immunoreactive rat insulin in the conditioned medium per $10^6$ cells per day (data not shown). No insulin was produced in cells transduced with a control retroviral vector, LX/hAAT (8).

**Tissue Distribution of LX/rINS After Portal Vein Infusion.** The procedure for retroviral-mediated transduction of rat hepatocytes *in vivo* involves a 70% partial hepatectomy followed 24 hr later by retroviral infusion into the portal vein (8). This results in 5–15% hepatocyte transduction and persistent gene expression for at least 6 months. The tissue distribution of the LX/rINS vector was determined by PCR analysis of genomic DNA using primers that specifically amplified the LX/rINS sequence. Of the eight tissues tested, the vector was detected in the liver at 0.01–0.1 copy per cell, corresponding to a 1–10% transduction frequency. The only alternative organ that was positive for LX/rINS sequences was the spleen, but only at 0.0001–0.001 copy per cell (Table 1). The remaining tissues contained <0.0001 copy of LX/rINS per cell, which was the level of detection of the assay. No LX/rINS vector sequences were detected in tissues from rats treated with the LX/hAAT vector or medium.

To determine if the LX/rINS was transcribed, mRNA from the liver and spleen was reverse transcribed followed by PCR analysis (rtPCR). LX/rINS specific transcripts were readily detected in the liver of LX/rINS-treated animals to a dilution of $10^{-4}$ (Table 2). Treatment of the mRNA with ribonuclease followed by rtPCR resulted in no product. No LX/rINS specific PCR product was detected in splenic mRNA, suggesting there were at least $10^4$-fold more LX/rINS transcripts in the liver.

**Induction of Diabetes in Rats and Insulin Gene Delivery to the Liver.** The efficiency of retroviral-mediated hepatic transduction in severely diabetic rats was low and variable, due to altered liver regeneration kinetics (10, 11). Thus, the hepatocytes of nondiabetic rats were transduced with LX/rINS, LX/hAAT, or medium, followed by induction of severe diabetes with a high dose of streptozotocin at 250 mg/kg (12). Three days after streptozotocin treatment, control rats lost ≈25% of their body weights while the average weight of LX/rINS-treated rats remained relatively constant (Fig. 1*A*). In 6 days, all 18 control rats died. In contrast, 13/16 rats transduced with LX/rINS survived for 21 days (Fig. 1*B*). Immunohistological staining of pancreatic sections with antibodies against rat insulin C peptide (Linco) revealed there was near-total ablation of β cells in all treatment groups 3 days after streptozotocin treatment (data not shown).

Table 1. Tissue distribution of LX/rINS

| Tissue | LX/rINS proviral sequences per rat haploid genome in various treatment groups: LX/rINS | LX/hAAT or medium |
|---|---|---|
| Liver | 0.01–0.1 | <0.0001 |
| Spleen | 0.0001–0.001 | <0.0001 |
| Other organs* | <0.0001 | <0.0001 |

$n$ = 3 for LX/rINS-treated rats; $n$ = 1 for LX/hAAT- and medium-treated rats.
*Kidney, pancreas, lung, heart, brain, and testes.

Table 2. Detection of LX/rINS-specific transcripts

| Tissue | Fold dilution of reverse-transcribed DNA: $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
|---|---|---|---|---|---|
| Liver | ++++ | ++++ | +++ | ++ | + |
| Spleen | – | ND | ND | ND | ND |

$n$ = 3 for liver and spleen mRNA samples. ND, not determined.

**Prevention of Ketoacidosis in LX/rINS-Treated Rats.** To determine what specific protective effect the LX/rINS vector was having, sera from all three groups of rats were tested for the presence of ketones. Three days after streptozotocin treatment all rats from the two control groups had serum ketone levels of 63 ± 21 mg/dl, which is considered high (12). However, all rats treated with LX/rINS had low or no serum ketones (Fig. 2*A*).

**Insulin, Glucagon, and C Peptide Production in LX/rINS-Treated Rats.** Rat sera were assayed for immunoreactive rat insulin by RIA. Fourteen days after retroviral transduction, nonfasting serum insulin levels in the control groups were 2.2 ± 1.8 ng/ml and those in rats transduced with LX/rINS were 7.1 ± 3.1 ng/ml (Fig. 2*B*). Three days after induction of diabetes, serum insulin levels in control rats decreased to 0.7

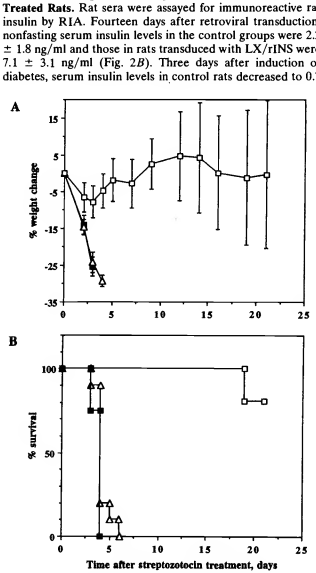


FIG. 1. Effect of streptozotocin treatment in experimental rats. (*A*) Percent weight change in rats following treatment with streptozotocin calculated from day 0. Each point was the average percent weight change ± SD. ■, Medium treatment group ($n$ = 8); △, LX/hAAT treatment group ($n$ = 10); □, LX/rINS treatment group ($n$ = 16). (*B*) Percent survival of rats in the three treatment groups.

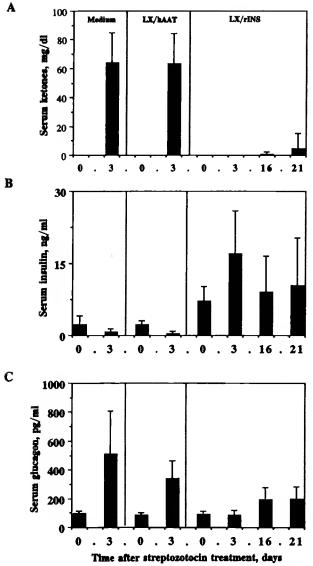


FIG. 2. Serum chemistry analyses of rats following treatment with streptozotocin. (*A*) Serum ketone levels were detected (by an Ames Ketostix) at the indicated times after induction of diabetes. (*B* and *C*) Serum insulin and serum glucagon levels, respectively. Each bar represents the mean ± SD for medium ($n$ = 8), LX/hAAT ($n$ = 10), and LX/rINS ($n$ = 16).

± 0.7 ng/ml, as expected, and those in rats treated with LX/rINS remained unchanged for 21 days (Fig. 2*B*).

Because high levels of immunoreactive insulin were detected in LX/rINS-treated rats, we tested if hepatically produced proinsulin was processed to mature insulin. The serum C peptide levels were determined concurrent with insulin levels. Since the half-life of mature insulin is shorter than that of C peptide (13), the C peptide-to-insulin (C:I) molar ratio in control rats ranged from 2.18 to 2.74 (Table 3), as expected (14). In LX/rINS-treated rats the ratio was 1.04 before streptozotocin treatment and was reduced to 0.22–0.47 after induction of diabetes (Table 3). The results indicated that hepatically produced proinsulin is not completely processed and is thus biologically less active (15).

Glucagon levels were similar in all treatment groups after retroviral transduction (Fig. 2*C*). Three days after induction of diabetes, however, glucagon levels in the control rats increased from 98 ± 18 pg/ml to 511 ± 296 pg/ml, as expected (16). The level remained normal for 3 days in rats transduced with the insulin vector, but there was an increase at 16 and 21 days (Fig. 2*C*). Thus, hepatic insulin was at least partially active in suppressing glucagon secretion.

Table 3. Plasma C:I molar ratios in diabetic rats

| Time after diabetes induction, days | C:I molar ratio | | |
|---|---|---|---|
| | Medium | LX/hAAT | LX/rINS |
| 0 | 2.74 ± 1.25 | 2.18 ± 0.20 | 1.04 ± 0.40 |
| 3 | 2.27 ± 1.24 | 1.63 ± 1.37 | 0.22 ± 0.08 |
| 16 | ND | ND | 0.41 ± 0.21 |
| 21 | ND | ND | 0.47 ± 0.39 |

$n$ = 8 for medium group, $n$ = 10 for LX/hAAT, and $n$ = 16 for LX/rINS. ND, not determined.

**Histopathological Examination of the Liver.** Histological examination of the liver 14 days after retroviral transduction showed no differences between rats transduced with LX/rINS, LX/hAAT, or medium (not shown). Insulin was not detected by immunohistological staining of the liver, which was expected since the liver does not store secretory proteins. Three days after the induction of diabetes, glycogen was absent in livers from control rats but was well preserved in rats trans-

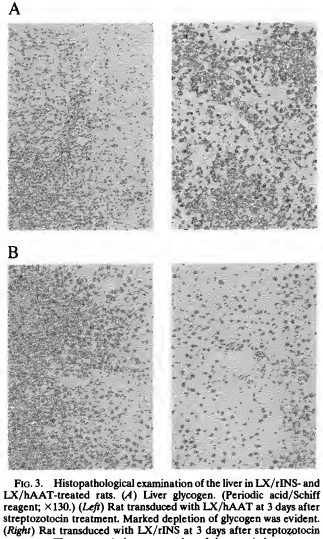


FIG. 3. Histopathological examination of the liver in LX/rINS- and LX/hAAT-treated rats. (*A*) Liver glycogen. (Periodic acid/Schiff reagent; ×130.) (*Left*) Rat transduced with LX/hAAT at 3 days after streptozotocin treatment. Marked depletion of glycogen was evident. (*Right*) Rat transduced with LX/rINS at 3 days after streptozotocin treatment. There was relative preservation of glycogen in hepatocytes. (*B*) Liver fat distribution 3 days after streptozotocin treatment. (Frozen section, oil red-O stain; ×130.) (*Left*) Rat transduced with LX/hAAT. Orange lipid droplets were present in most hepatocytes. (*Right*) Rat transduced with LX/rINS. There was no lipid detected.

duced with LX/rINS (Fig. 3A). Also, by day 3, small intracellular fat inclusions were evident in most of the hepatocytes in liver sections from control rats, which were absent in rats transduced with the insulin vector (Fig. 3B).

**Blood Glucose Levels in LX/rINS-Treated Rats.** To determine if the high immunoreactive insulin levels posed a danger to the rats, 24-hr blood glucose profiles were established at 2-hr intervals. Fourteen days after retroviral transduction, nonfasting blood glucose levels of all rats remained constant at ≈100 mg/dl throughout the 24-hr period. Three days after induction of diabetes, rats transduced with either the control vector or insulin vector had similarly elevated nonfasting levels of blood glucose to >250 mg/dl, which persisted for the entire 24-hr period (Fig. 4). Upon fasting, however, the blood glucose levels in control rats remained elevated (>250 mg/dl) for the first 10 hr and then slowly decreased over the next 14 hr (Fig. 4A). The fasting blood glucose levels in the LX/rINS-treated group, however, decreased to normoglycemic levels (90–110 mg/dl) within the first 4 hr of the fast and remained in this range for 20 hr (Fig. 4B). At no point during the 24-hr fast did the blood glucose level of any of the LX/rINS-treated rats decrease to <50 mg/dl, which is considered hypoglycemic (4).

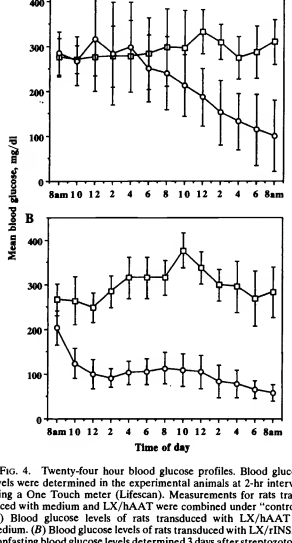


FIG. 4. Twenty-four hour blood glucose profiles. Blood glucose levels were determined in the experimental animals at 2-hr intervals using a One Touch meter (Lifescan). Measurements for rats transduced with medium and LX/hAAT were combined under "control." (A) Blood glucose levels of rats transduced with LX/hAAT or medium. (B) Blood glucose levels of rats transduced with LX/rINS. □, Nonfasting blood glucose levels determined 3 days after streptozotocin treatment; ○, fasting blood glucose levels determined 5 days after streptozotocin treatment. The results are mean ± SD. In the LX/rINS-treated group, $n = 9$; for control rats under nonfasting conditions, $n = 16$; under fasting conditions $n = 8$.

## DISCUSSION

Results of this investigation suggest that the liver is an excellent target organ for ectopic insulin gene expression as a potential treatment modality for prevention of ketoacidosis in severe diabetes mellitus. This conclusion is supported by previous reports that transplantation of β cells into the liver, or hepatic insulin expression in transgenic mice (17), alleviates the effects of severe diabetes. Recombinant retroviral transduction of the liver *in vivo* has achieved a low-level insulin gene expression that is sustained over time. This insulin activity prevented ketoacidosis and death associated with severe diabetes in rats induced by streptozotocin administration. Normoglycemia was also achieved in the treated animals during a 24-hr fast. There is no evidence of any adverse effects of hepatic insulin gene expression in the treated animals, which confirms previous reports that insulin production from transplanted β cells in the liver (18, 19), or insulin expression from the liver of transgenic mice (17), did not cause liver damage or abnormal biology.

Infusion of the retrovirus into the portal vein results in preferential transduction of the liver. The level of transduction with the LX/rINS vector estimated by the PCR analysis (1–10%) is within range of the transduction efficiency of 5–15% determined using a β-galactosidase reporter gene (8). Since no significant level of LX/rINS provirus, or LX/rINS-specific transcript, was detected in tissues other than the liver, it may be concluded that the major source of ectopically produced insulin is the liver.

Treatment of the rats with a high dose of streptozotocin resulted in near-total destruction of pancreatic β cells in all rats (20). Consequently, within 4 days of treatment, serum insulin levels in control rats decreased to <0.2 ng/ml, the detection level of the assay (unpublished results). Therefore, insulin secretion by residual β cells is inconsequential. There was no appreciable change in serum insulin levels in rats treated with the insulin vector, suggesting an extrapancreatic source of insulin production. There are several indications that this procedure is efficacious in controlling severe diabetes. (*i*) Insulin inhibits the production of glucagon (16). Although glucagon levels in control diabetic rats increased severalfold, as expected, it remained in the normal range in rats transduced with the insulin vector. (*ii*) Low serum insulin and high glucagon levels promote ketoacidosis, which was evident in the control diabetic rats that all died in 4–6 days. Rats treated with LX/rINS, however, had no significant blood ketones and 13/16 survived for 21 days. (*iii*) Sufficient insulin activity was present in animals treated with the insulin vector to have prevented other catabolic activity in the liver such as glycogen breakdown, triglyceride accumulation, and ketogenesis (21, 22). Collectively, the results suggest that the acute clinical conditions associated with severe diabetes were prevented in rats treated with the insulin vector.

Unregulated low-level insulin gene expression from the liver does not appear to cause life-threatening hypoglycemia under the conditions of our study. Under nonfasting conditions, there was no difference in blood glucose profiles in all rats before and after induction of diabetes. Normoglycemia was achieved during a 24-hr fast in LX/rINS-treated rats with no evidence of hypoglycemia, which is consistent with fasting levels of insulin activity being present in these animals. Blood glucose levels in control rats also decreased 12 hr into the fast. This observation is consistent with reports that in human ketoacidotic diabetic patients, blood glucose levels decrease to normoglycemic levels under fasting conditions (23).

Three rats in the insulin vector treatment group died 19 days after streptozotocin administration and the remaining treated animals succumbed a few days afterward. The experimental

rats had low serum ketone levels, and the livers showed no fat deposition and no glycogen depletion, which strongly suggests that the primary cause of death was not worsening diabetes. Elevated creatinine and blood urea nitrogen (BUN) levels suggested the rats succumbed to kidney failure, possibly caused by streptozotocin, a known nephrotoxin at high doses (24). Histological examination revealed diffusely damaged proximal tubules that were strikingly different from the lesions observed in the kidneys of control rats dying from acute ketoacidosis. There is no indication that the LX/rINS vector did contribute to the kidney failure since no vector sequences were detected in the kidneys of LX/rINS rats, and creatinine and BUN levels were unchanged 14 days after vector introduction.

Although low levels of insulin activity were evident in LX/rINS-treated rats, the level of immunoreactive insulin was 5-fold higher than in normal rats. An altered C:I molar ratio suggested hepatically produced proinsulin was not fully processed to mature insulin and was thus biologically less active (15). Hepatocytes, like other cell types with a constitutive secretory pathway, proteolytically process secretory proteins by the enzyme furin, which cleaves at specific amino acid sequences (25). Furin will properly process rat 1 proinsulin to mature insulin if overexpressed in COS cells (26) or if the cleavage site in proinsulin is converted to the furin recognition sequence (27). Expression of these engineered insulin genes in the liver of diabetic animals should result in the production of mature insulin that will permit the reduction of recombinant viral doses in future experiments. Even without complete processing, we have demonstrated unambiguously that a sustained low level of insulin activity can prevent the acute lethal consequences of IDDM in rats. The implications of this research extend past the treatment of IDDM. Recent studies have shown that prophylactic insulin injected into human patients predisposed to developing IDDM delays or prevents disease onset (28). With the development of better vectors for insulin gene delivery, regulation of its expression, and processing *in vivo*, severe diabetes can be treated in the future by insulin production in ectopic tissues through somatic gene therapy.

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# Preproglucagon Gene Expression in Pancreas and Intestine Diversifies at the Level of Post-translational Processing*

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**Glucagon is a pancreatic hormone of 29 amino acids that regulates carbohydrate metabolism and glicentin is an intestinal peptide of 69 amino acids that contains the sequence of glucagon flanked by peptide extensions at the amino and carboxy termini. The glucagon gene encodes a precursor containing glucagon and two additional, structurally related, glucagon-like peptides separated by an intervening peptide. These peptides are encoded in separate exons. To determine whether the pancreatic and intestinal forms of glucagon arise by alternative RNA and/or protein processing, we used antisera to synthetic glucagon-like peptides and exon-specific, complementary oligonucleotides for analyses of proteins and mRNAs in pancreatic and intestinal extracts. Preproglucagon mRNAs are identical, but different and highly specific peptides are liberated in the two tissues. Immunocytochemistry shows colocalization of glucagon and the two glucagon-like peptides in identical cells. We conclude that diversification of preproglucagon gene expression occurs at the level of cell-specific post-translational processing.**

The diversification of genetic information can take place at one or more of several levels of gene expression (Darnell, 1982): 1) gene duplication and divergence, 2) alternative splicing of RNA precursors, and 3) alternative processing of polyproteins. The genes encoding peptide hormones are exemplary in the utilization of a variety of levels in gene expression at which to amplify a genetic repertoire of informational molecules. For example, during evolution the parent genes for growth hormone and insulin have expanded into supra-gene families (Dayhoff, 1978; Seeburg, 1982). Alternative splicing of RNA precursors transcribed from the calcitonin (Rosenfeld *et al.*, 1983) and tachykinin (Nawa *et al.*, 1983) genes each generate two mRNAs with substituted exons (exon-switching); the exons encode either the peptide calcitonin or an alternate calcitonin-related peptide and either substance P or substance K plus substance P. A 19-base insertion/deletion splicing variation in the processing of the pre-mRNA encoding the precursor of gastrin-releasing peptide leads to a frame-shift in the protein-coding sequence giving rise to two different prohormones (Spindel *et al.* 1985). Alternative patterns of the post-translational processing of pro-opiomelanocortin apparently accounts for the different adenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (MSH) peptides observed in anterior and intermediate lobes of the pituitary (Zakarian and Smyth, 1982; Roberts *et al.*, 1978).

These modes for the diversification of genetic information at each of the three levels of gene expression are highly tissue- and cell-specific. Diversification of expression of genes encoding peptide hormones appears to provide the multiple, diverse regulatory functions of the peptides served in the capacity of neuromodulators (Krieger, 1983), metabolic hormones (Habener, 1985b), immune regulators (Blalock et al., 1985), and cellular differentiation factors (Hanley, 1985).

Studies of the structure and regulation of the glucagon gene provide an opportunity to explore the potential diversification of information at all of the levels of gene expression described above. Glucagon, a peptide hormone that regulates glycogenolysis and gluconeogenesis in the liver (Lefebvre, 1983; Unger and Orci, 1981; Habener, *et al.*, 1985a), is a member of a multigenic family of several structurally related peptides that exert physiologic actions in the nervous and gastrointestinal systems. The 29-residue glucagon is produced by the endocrine pancreas and glicentin, a larger peptide of 69 amino acids that contains the entire sequence of glucagon, is produced in the intestines (Sundby *et al.*, 1976; Thim and Moody, 1981; Unger and Orci, 1981).

Pulse-labeling studies of rat pancreatic islets have shown that glucagon is synthesized as a precursor, proglucagon, (Tager and Steiner, 1973; Patzelt *et al.*, 1979; Patzelt and Schiltz, 1984). Complementary DNAs prepared from hamster (Bell *et al.*, 1983a), bovine (Lopez *et al.*, 1983), and rat (Heinrich *et al.*, 1984a) pancreas encode pancreatic glucagon in preproglucagon, a precursor of 180 amino acids (Fig. 1). Preproglucagon contains in tandem, and separated by intervening peptides, the sequences of glucagon and two cryptic peptides of 37 and 33 amino acids, termed glucagon-like peptides (GLPs[1]) I and II. Glucagon and each of the two glucagon-like peptides is flanked by pairs of basic amino acids that are characteristics of the sites that are cleaved during the post-translational processing of prohormones resulting in the liberation of specific bioactive peptides (Steiner *et al.*, 1980). The gene encoding pancreatic preproglucagon consists of six exons, the internal four of which individually encode the signal peptide, glucagon, GLP-I, and GLP-II (Bell *et al.*, 1983b; Heinrich *et al.*, 1984b).

The close homology of the amino-terminal region of pancreatic proglucagon and glicentin, and the encodement of glucagon and the two glucagon-like peptides in separate exons of the pancreatic preproglucagon gene, raises important ques-

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[1] The abbreviations used are: GLP, glucagon-like peptide; IP, intervening peptide; HPLC, high pressure liquid chromatography.

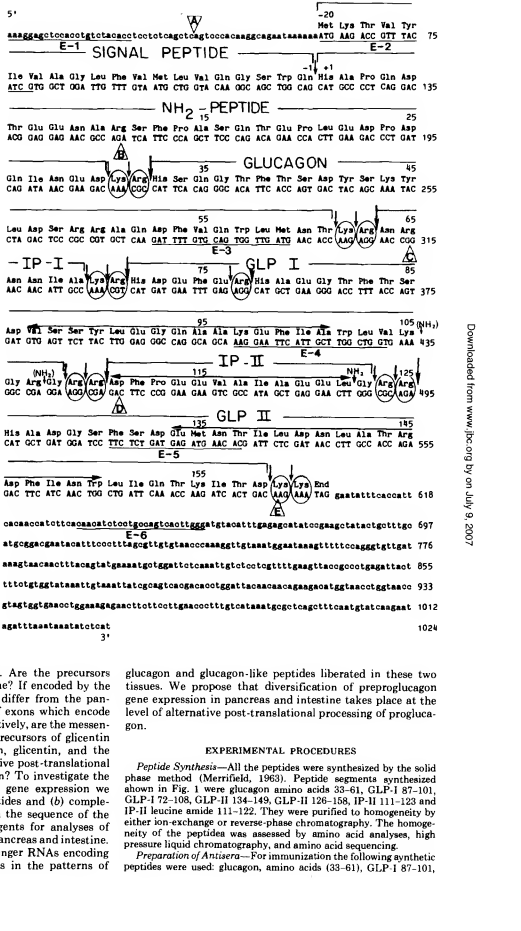


FIG. 1. **Sequence of rat preproglucagon.** The signal sequence and the internal functional peptides predicted by their homology with glucagon and separated by pairs of circled basic amino acids (lysine, arginine) are indicated. *Solid overlines* designate regions of sequence that were synthesized for immunization and preparation of antisera. *Underlines* (*E1–E6*) designate regions of nucleotide sequence to which complementary oligonucleotides were synthesized. *Arrows* indicate sites of enzyme cleavages utilized in the processing of the preproglucagon. *Parentheses around arrows* denote potential and not proven cleavages. $NH_2$ = amidated amino acid. *Triangles* encoded *A–E* denote positions of introns in the gene (Heinrich *et al.*, 1984b).

tions about glucagon gene expression. Are the precursors encoded by a separate or the same gene? If encoded by the same gene, does the glicentin mRNA differ from the pancreatic glucagon mRNA by deletion of exons which encode the two glucagon-like peptides? Alternatively, are the messenger RNAs and therefore the encoded precursors of glicentin and glucagon the same, and glucagon, glicentin, and the glucagon-like peptides arise by alternative post-translational processing of a common preproglucagon? To investigate the mechanism of tissue-specific glucagon gene expression we prepared (*a*) antisera to synthetic peptides and (*b*) complementary oligonucleotides deduced from the sequence of the rat glucagon gene and used these reagents for analyses of mRNAs and proteins in extracts of rat pancreas and intestine. We find similar, if not identical, messenger RNAs encoding preproglucagon and marked differences in the patterns of glucagon and glucagon-like peptides liberated in these two tissues. We propose that diversification of preproglucagon gene expression in pancreas and intestine takes place at the level of alternative post-translational processing of proglucagon.

## EXPERIMENTAL PROCEDURES

*Peptide Synthesis*—All the peptides were synthesized by the solid phase method (Merrifield, 1963). Peptide segments synthesized ahown in Fig. 1 were glucagon amino acids 33–61, GLP-I 87–101, GLP-I 72–108, GLP-II 134–149, GLP-II 126–158, IP-II 111–123 and IP-II leucine amide 111–122. They were purified to homogeneity by either ion-exchange or reverse-phase chromatography. The homogeneity of the peptides was assessed by amino acid analyses, high pressure liquid chromatography, and amino acid sequencing.

*Preparation of Antisera*—For immunization the following synthetic peptides were used: glucagon, amino acids (33–61), GLP-I 87–101,

GLP-II 134–149, IP-II leucine amide 111–122 and GLP-I 1–37 (72–108); the latter was purchased from Bachem.

Antisera were produced in New Zealand White Rabbits utilizing the immunization schedule of Vaitukaitis and Ross (Vaitukaitis *et al.*, 1971). For each peptide five 6-week-old rabbits were injected with an antigen mixture at 10–20 sites intradermally in their backs at three consecutive weekly intervals. The antigen mixture consisted of 50 μg of peptide first mixed with saline and 100 μg of methylated albumin and then emulsified with an equal amount of complete Freund's adjuvant. Thus, once a week for 3 consecutive weeks each animal received 50 μg of peptide in multiple intradermal sites. Starting 6 weeks after the third and final immunization 20-40 ml of heparinized blood was collected at biweekly intervals by arterial puncture of the ear. Plasma prepared from the blood was stored frozen for subsequent evaluation of specific antibody titers and competitive binding radioimmunoassays to check for cross-reactivity of the glucagon and GLP antisera amongst the peptides.

*Radioimmunoassay*—Determinations of titers of specific antibodies and the preparation of competitive binding radioimmunoassays were carried out using the procedure described previously (Habener *et al.*, 1976). Peptides containing tyrosine residues were labeled with $I^{125}$-iodine using Iodogen (Rojos *et al.*, 1983). Iodinated peptides were isolated by gel filtration on Biogel P-10 columns (1 × 15 cm), and their homogeneity was checked on high pressure reverse-phase chromatography on a C-18 column. Incubations of antisera with labeled and unlabeled peptides was carried out in conditions of nonequilibrium for 48 h (24 h without added labeled peptide followed by 24 h after labeled peptide was added). Radioimmunoassay buffer consisted of 0.15 M boric acid, 0.25% bovine serum albumin, 0.005 M EDTA, 0.01 M benzamidine, and 0.1% sodium azide at a final pH of 8.0. Antisera, buffer, and sample were incubated at 4 °C in a final volume of 0.5 ml. Antibody-bound and unbound peptides were separated by absorption of the unbound peptides to dextran-coated charcoal. Assay results were expressed as the ratios of amounts of radioactivity of antibody bound to free peptide ($B/F$).

*Immunocytochemistry*—Normal adult rats were fixed by vascular perfusion with 1% glutaraldehyde in 0.1 M phosphate buffer. Pieces of pancreas and gastrointestinal tract were dehydrated in ethanol and embedded in Epon 812. Consecutive semithin (0.5–1 μm) sections of pancreatic islets and ileal mucosa were processed by the indirect immunofluorescence technique (Coons *et al.*, 1955). The following antisera and dilutions thereof were used: a C-terminally directed antiglucagon (K6251, a gift of L. Heding, Denmark) diluted 1:200, anti-glicentin (R64, a gift of A. J. Moody, Denmark) diluted 1:100, anti-GLP 1 (1:100), anti-IP II (1:100), and anti-GLP II (1:100). Incubations with the immune sera, or control incubations with immune serum preabsorbed with 5–10 μg of peptide, were carried out for 2 h at room temperature. After washing with phosphate-buffered saline, sections were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 h at room temperature. Sections were washed with phosphate-buffered saline, counter-stained with Evans blue, and observed in a Leitz ortholux microscope equipped with a pleomopak 2.2 illuminator.

*Electron Microscopy*—Thin sections of rat and human pancreas embedded at low temperatures in Lowicryl were collected on nickel grids. Incubation with the antisera were carried out overnight at 4 °C in a moist chamber. Antiserum to GLP-1 (1–37) was used at a dilution of 1:500. After washing with distilled water, sections were treated with the protein A-gold solution (gold particles ~15 nm) for 1 h at room temperature, then rinsed with distilled water, and double-stained with uranyl acetate and lead citrate before examination with the electron microscope.

*Extraction of Tissues*—Eight 250–300 g male rats were used: four were fasted overnight and four had free access to food. The rats were killed by decapitation and pancreas, small intestine, and large intestine were rapidly excised (within 30 s). To minimize degradation and to maximize recovery of peptides, two procedures were used in acidic conditions to extract glucagon-related peptides from rat pancreas and intestines. 1) the tissues were instantly frozen in dry ice, pulverized to a powder with mortar and pestle, and extracted by trituration with 8 M urea, 0.2 N HCl. 2) the tissues were immediately homogenized in a polytron with 8 M urea, 0.2 N HCl. The homogenates were clarified by centrifugation of 12,000 × *g* for 30 min. Supernatants were collected and were neutralized with NaOH before radioimmunoassay and chromatography. We have shown previously that extracts of tissues in acid-urea prevents actions of cathepsins that cleave cogmate prohormones (Habener *et al.*, 1976).

*Gel Filtration Chromatography*—Solutions of tissue extracts of pancreas, small and large intestine in 6 M urea, 0.2 N HCl (2–3 ml) were applied onto Biogel P-30 columns (1.6 × 90 cm) that were equilibrated in the radioimmunoassay buffer: 0.15 M borate (pH 8.2) containing 0.25% bovine serum albumin, 10 mM benzamidine, 5 mM EDTA, and 1:10,000 final concentration merthiolate. The samples were eluted at a flow-rate of 5 ml/h and collected in 2.2-ml fractions. As a marker of the elution positions of samples [$^{14}$C]glycine (~100,000 cpm) was added in each of the solutions of tissue extracts or the mixture of standards. Each tissue homogenate was run on a different column. A mixture of glucagon and synthetic GLP-I (1–37), GLP-II (1–33), and IP-II amide (250 μg of each in 1 ml radioimmunoassay buffer) containing the [$^{14}$C]glycine marker was used to calibrate the elution positions of the gel filtration columns. Aliquots of fractions of column eluate collected were analyzed by radioimmunoassay with antisera to glucagon, GLP-1, GLP-II, and IP-II in triplicate each of 10 and 100 μl.

*High Pressure Liquid Chromatography*—Further analysis was accomplished by either ion-exchange or reverse-phase HPLC. The reverse-phase chromatography was on Radial-Pak μBondapak C-18 (8 mm × 10 cm) cartridge. Solvent systems were as follows: A, 90% water containing 0.1% trifluoroacetic acid, 10% acetonitrite, and B, 10% water containing 0.1% trifluoroacetic acid, 90% acetonitrite. Resolution of glucagon, GLP-I (1–37), GLP-II (1–33), and IP-II amide was accomplished with a linear gradient of 20–70% B formed in 20 min at a flow-rate of 5 ml/min.

Aliquot (1.0 ml) from a fraction that immediately followed the individual peak of immunoreactivity from the gel filtration column was applied onto the HPLC column through a manual injector. The outlet of the column was connected to a fraction collector, and 2.5-ml fractions were collected. Samples were dried under vacuum in a speed-vac and reconstituted with the radioimmunoassay buffer (1.0 ml). 10- and 100-μl aliquots were analyzed in triplicate by radioimmunoassay.

Peak fractions collected from gel filtration were also analyzed by ion-exchange on a Protein-Pak DEAE 52 (7.5 mm × 7.5 cm). Solvent systems were: A, 0.02 M Tris-HCl (pH 8.5) and B, 0.02 M Tris-HCl (pH 8.5) containing 0.5 M NaCl. Synthetic standards of GLP-I (7–37) and GLP-I (1–37) were resolved from each other by a linear gradient of 0–70% B formed in 25 min at 1.0 ml/min. The synthetic standards of carboxylated and amidated IP-II were separated by a linear gradient of 20–70% B formed in 30 min at flow-rate of 1 ml/min. Column eluates were collected in 1-ml fractions in connical plastic test tubes containing 100 μl of 0.02 M Tris-HCl (pH 8.5), 2.5% bovine serum albumin. Aliquots (10 and 100 μl) were analyzed directly in triplicate by radioimmunoassay.

*Preparation of RNA*—Adult Sprague-Dawley rats (Charles River

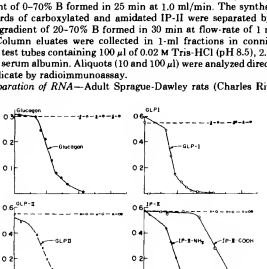


FIG. 2. **Complete specificity of radioimmunoassays for peptides processed from proglucagon.** Curves shown for increasing amounts of peptide measure displacement of $^{125}$I-labeled peptide corresponding to each assay (see "Experimental Procedures"). Antisera were prepared to: glucagon 1–29 (33–61), GLP-1 1–37 (72–108), subpeptides to GLP-I (87–101) and GLP-II (134–149), and IP-II amide (111–122) where numbers in parentheses refer to sequence positions shown in Fig. 1. Antiserum used in assay for GLP-I shown is sensitive for detection of processed peptides but has poor sensitivity for the detection of proglucagon and other precursor forms of glucagon. (●) glucagon, (○) GLP-1, (×) GLP-II, (△) IP-II leucinamide, (□) IP-II glycine COOH.

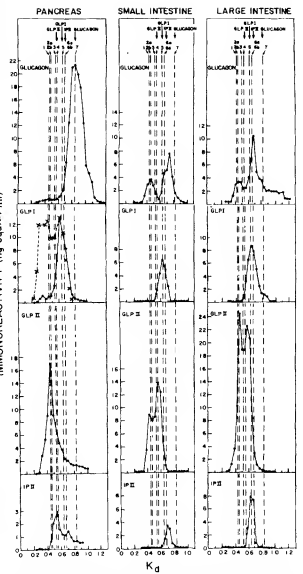


FIG. 3. **Tissue-specific processing of proglucagon into glucagon and glucagon-like peptides.** *Panels* show chromatograms from Bio-Gel P-30 columns of extracts of rat pancreas, small intestine, and large intestine. Aliquots of the identical fractions were assayed by specific radioimmunoassays for the peptides (see Fig. 2). All tissues shown were from a single rat. Analyses were carried out on tissues from two additional rats with identical results. *Arrows* indicate elution positions of synthetic peptides glucagon 1–29 (33–61), GLP-I 1–37 (72–108), GLP-II 1–33 (134–158), and IP-II leucine amide (111–122) where *numbers in parentheses* designate sequence positions shown in Fig. 1. *Numbers* identifying *vertical dashed lines* refer to specific peaks of immunoreactivity, some of which were further analyzed by HPLC and radioimmunoassay. *Ordinate values* of immunoreactivity are based on assay standards of the above described peptides. GLP-I values shown in *closed circles* are from assay using the antiserum sensitive for the detection of processed GLP-1 peptides and insensitive for detection of proglucagon and precursor forms of glucagon. Values shown in (×) are from assay using the antiserum that readily detects proglucagon as well as processed GLPs. Column volume of 1.0 $K_d$ indicates position of elution of [$^{14}$C]glycine.

Breeding Laboratories, Wilmington, MA) were killed by decapitation, and the pancreas and large intestine were removed. Total RNA was extracted immediately from these fresh tissues by the guanidinium-thiocyanate method (Glisin *et al.*, 1974) and further purified by centrifugation through 5.7 M CsCl (Heinrich *et al.*, 1983; Chirgwin *et al.*, 1979). Poly (A$^+$) RNA was prepared from total RNA by chromatography on oligo(dT)-cellulose.

*Preparation of $^{32}$P-labeled Complementary Oligonucleotides*—Oligonucleotides were synthesized by the phosphoamidite method using an automated DNA Synthesizer (Applied Biosystems Inc., 380A, Foster City, CA) and 5′ end-labeled with $^{32}$P using [$\gamma$-$^{32}$P]ATP (7000 Ci/mol, ICN) and T$_4$ polynucleotide kinase (Bethesda Research Laboratories). The phosphorylated oligonucleotides were separated from unincorporated [$\gamma$-$^{32}$P]ATP by ion-exchange chromatography (Elutip, Schleicher & Schuell).

*Northern RNA Analyses*—Aliquots (1 $\mu$g) of pancreatic, and aliquots of intestinal poly (A$^+$) RNA (5 $\mu$g) were denatured in 50% dimethyl sulfoxide (w/v), 6% formaldehyde, 10 mM morpholine (pH 7.4) 0.05% bromphenol blue (w/v), and xylene cyanol (w/v) for 15 min at 65 °C. The denatured RNA was size-fractionated by electrophoresis through 1.5% agarose gel containing 6% formaldehyde. Fractionated RNA was transferred to nylon membranes (Gene Screen, New England Nuclear) by electrophoresis. The membranes were hybridized with oligonucleotides in 1 M NaCl, 1% sodium dodecyl sulfate at 45 °C for 16 h, and washed in 0.3 M NaCl, 0.1% NaDodSO$_4$ at room temperature. Autoradiography of the membrane was performed at −70 °C with a Cronex Lightning Plus intensifying screen. Quantitative analysis of the autoradiograms was performed with a Quick-Scan densitometer as described previously (Heinrich *et al.*, 1983).

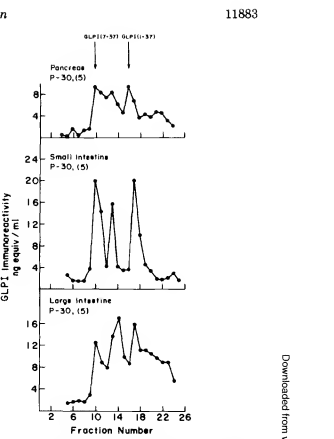


FIG. 4. **Glucagon-like peptide I consists of several distinct forms.** High pressure liquid chromatography was performed on the major GLP-1 immunoreactive components from P-30 gel filtration (*peak 5*, Fig. 3) of extracts of pancreas and intestines. Chromatography was on a DEAE-52 ion-exchange column (see "Experimental Procedures"). Fractions were analyzed for GLP-1 immunoreactivity by GLP-I-specific radioimmunoassay using antiserum sensitive for detection of processed GLPs (Fig. 3). Recoveries of immunoreactivity were 80–90%.

## RESULTS

*Strategies for the Selection of Peptides for Chemical Synthesis*—Peptides synthesized were as follows (see Fig. 1): two separate glucagon-like peptide I (residues 72–108 and 87–

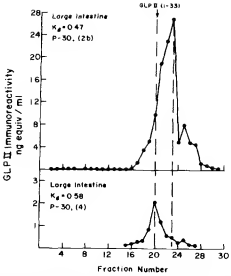


FIG. 5. **Large intestine contains authentic GLP-II (1–33) and a large unidentified peptide.** High pressure liquid chromatography was carried out on a μBondapak C-18 reverse-phase column with the two peak positions of GLP-II detected by gel filtration (P-30) and radioimmunoassay (*peaks 2* and *4*, Fig. 3). See "Experimental Procedures" for details. Radioimmunoassay was specific for detection of GLP-II determinants (Fig. 2).

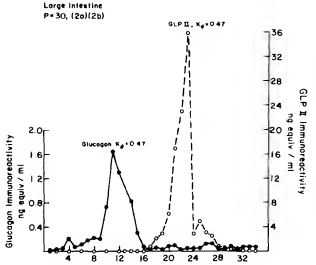


FIG. 6. **Glucagon and glucagon-like peptide II immunodeterminants in peak 2 of gel filtration (P-30) analysis of intestinal extract reside in separate peptides.** The peak 2 fraction ($K_d$ = 0.47) was analyzed by HPLC on a reverse-phase column. Fractions were assayed by radioimmunoassays specific for glucagon and GLP-II (Fig. 2).

101), intervening peptide II with an amidated carboxy-terminal leucine (residues 111–122) and glucagon-like peptide II (residues 134–149). The subpeptides of glucagon-like peptides I and II were chosen for syntheses because they contain the least similarities in amino acid sequences to glucagon and the corresponding glucagon-like peptides, a finding typical of the sequence diversification that takes place in the carboxy-terminal regions of the various peptides in the glucagon family. The sequence for the intervening peptide II of a carboxy-terminal leucineamide was deduced from the proteolytic cleavage sites at the basic residues located at positions 109–110

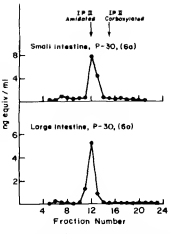


FIG. 7. **Intervening peptide II in intestinal extracts corresponds to the carboxy-terminal amidated (leucineamide) peptide.** The peak of IP-II immunoreactivity from P-30 gel filtration (*peak 6*, Fig. 3) was analyzed by HPLC on a DEAE-52 ion-exchange column. The peak of immunoreactivity determined by IP-II specific radioimmunoassay (Fig. 2) corresponds to elution position of synthetic amidated IP-II and not carboxylated IP-II.

and 124–125 following the glycine residue at position 123. Because it has been shown that a glycine preceding basic residues that are cleaved during the post-translational processing of prohormones serves as a donor of an amide function to the adjacent terminal amino acid, particularly if the amino acid is hydrophobic (Bradbury *et al.*, 1982), we assumed that the leucine position 122 would be amidated during the processing of the proglucagon. Inasmuch as both the intervening peptide II and the subpeptide of glucagon-like peptide II did not contain tyrosines for radioiodination two additional peptides were synthesized with the addition of amino-terminal tyrosine residues.

*Specificity of Peptide Antisera to Various Domains of Proglucagon*—The antisera produced to the peptides were specific for detection of their homologous antigens; no cross-reactivity was detectable among glucagon, GLP-I, GLP-II, and IP-II in a competitive binding radioimmunoassay (Fig. 2). The antisera were evaluated at the following different dilutions: in radioimmunoassays (1:500 for glucagon, 1:10,000 for GLP-I, 1:800 for GLP-II, and 1:2,000 for IP-II) and at 1:50 for antisera used in immunocytochemical studies. For the detection of GLP-I immunoreactivity, an antiserum to GLP-I 1–37 (72–108) and the carboxyl peptide (87–101) was used in the radioimmunoassay and immunocytochemical analyses. Only the antisera to GLP-I 1–37 (72–108) readily detected amounts of GLP-I immunoreactivity in extracts of rat pancreas and intestines.

To test the specificity of the IP-II antibody toward the amidated carboxy-terminal portion of the molecule, we also synthesized the sequence of the intervening peptide II that contains the glycine residue as the carboxy-terminal (residues 111–123). In the competitive binding radioimmunoassay the antiserum showed a 1000-fold reduced sensitivity toward carboxylated compared to amidated intervening peptide II (Fig. 2).

*Gel Filtration Analyses of Glucagon-like Peptides in Extracts of Pancreas and Intestines*—Marked differences are evident in the chromatographic patterns of specific immunoreactive peptides contained in extracts of pancreas and intestines (Fig. 3). The most striking differences in peptide patterns are in the relative amounts of glucagon, glucagon-like peptide II, and the intervening peptide II in pancreas and intestines.

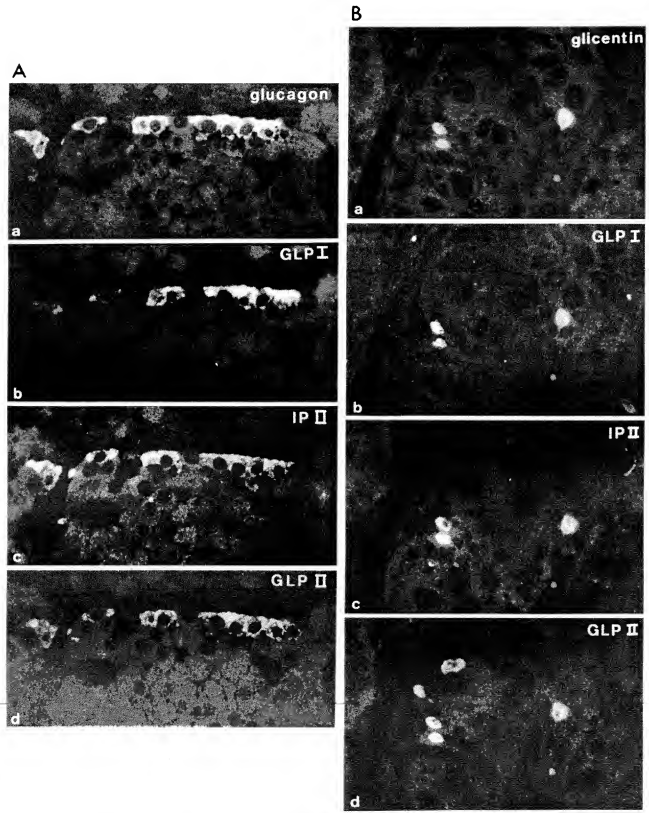


FIG. 8. **Consecutive semithin sections through (*A*) the periphery of a rat islet and (*B*) rat ileal mucosa.** The same cells are immunolabeled after indirect immunofluorescence staining with antisera against C-terminal glucagon (or glicentin) (*a*), GLP-I (*b*), IP-II (*c*), and GLP-II (*d*). Antiserum to GLP-I is sensitive for detection of processed peptides (Fig. 2).

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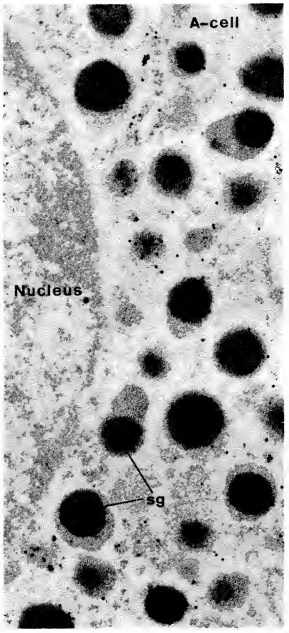



FIG. 9. **Electron microscopy of a human pancreatic A-cell treated with antiserum to glucagon-like peptide 1 (prepared by immunization with the full-length peptide, 1–37) conjugated and detected by the protein A-gold technique (Roth *et al.*, 1978).** Gold particles are localized over the dense core of the *a* secretory granules (*sg*). Magnification is × 39,500.

Essentially all of the glucagon immunoreactivity in the pancreas is represented as glucagon (*peak 7*) whereas, in the intestine, virtually no glucagon is present. Instead the intestinal glucagon immunoreactivity appears in forms of higher molecular weight that, based on earlier studies (Baldissera and Holst, 1984), are likely to consist of glicentin (*peak 2a*) and oxyntomodulin (*peak 6a*). Inasmuch as it is known that glicentin constitutes most of the intestinal glucagon, it is likely that our antiserum, as do most antisera to glucagon, (Conlon, 1980) underestimates the amounts of glicentin present by approximately 10-fold.

Likewise, there are remarkable qualitative differences between pancreas and intestinal extracts in the GLP-II and IP-II peaks of immunoreactivity. In the intestines a large peak of immunoreactivity coelutes in the position of GLP-II (*peak 4*) as well as a peak of higher molecular weight (*peak 2b*). In contrast, essentially all of the GLP-II immunoreactivity in pancreas is large (*peak 1*). Similarly, in the intestines distinct peaks of IP-II elute in the position of the synthetic IP-II-amide (*peak 5*), but in the pancreas the small amounts of IP-II immunoreactivity detected are larger than IP-II-amide (*peak 3*). In both pancreas and intestines, a major peak of GLP-I immunoreactivity elutes at the position of synthetic GLP-1 (1–37) (*peak 5*). In extracts of pancreas, but not intestines, a large form of GLP-1 is detected by a radioimmunoassay using an antiserum which is more sensitive for the detection of proglucagon than is the antiserum that detects GLP-1 (1–37).

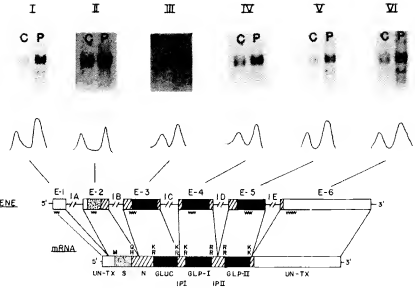
Quantitatively, in the pancreas both glucagon and GLP-I are liberated from proglucagon, and in the intestine GLP-I, GLP-II, and IP-II are liberated as discrete peptides.

*Analyses by High Pressure Liquid Chromatography and Radioimmunoassay of Selected Fractions Obtained from Gel Filtration*—To further characterize the glucagon-like peptides in extracts of pancreas and intestines, peak fractions of immunoreactivity obtained from the gel filtration analyses shown in Fig. 3 were analyzed by HPLC or an ion-exchange column (DEAE-52). The peak 5 fraction (Fig. 3) of GLP-I immunoreactivity resolved into several distinct peaks (Fig. 4). Two of the peaks coeluted precisely with the synthetic standard peptides GLP-1 (1–37) and GLP-1 (7–37) corresponding to the sequence positions 72–108 and 78–108 of Fig. 1, respectively. Similar analyses of the peak 5 fraction by HPLC on a C-18 hydrophobicity column gave only a single peak of immunoreactivity; the separation of GLP-I into the multiple peaks was only seen with the ion-exchange chromatography. Thus, in addition to the formation of the full-length GLP-I (1–37) processing also occurs at the arginine position 77 (Fig. 1) to give GLP-I (7–37).

Analyses by HPLC reverse-phase chromatography of the two peaks (*2b* and *4*; Fig. 3) in the large intestine revealed comigration of the peak 4 but not peak 2b with the synthetic GLP-II standard peptide (Fig. 5). We therefore conclude that GLP-II (1–33) is liberated from proglucagon in the intestines and that a substantial amount of GLP-II immunoreactivity exists in a different form, most likely a partially processed proglucagon (Fig. 3). To determine whether the glucagon and GLP-II immunoreactive moieties that elute in the peak 2 of high molecular weight consist of a single molecule containing both glucagon and GLP-II immunodeterminants, peak 2 was analyzed by reverse-phase HPLC (Fig. 6). The two determinants reside in two distinct peptides both of which differ from glucagon (1–29) and GLP-II (1–33) and likely consist of partially processed forms of proglucagon.

Analyses of the IP-II immunoreactivity of peak 6 (Fig. 3) in the intestinal extracts demonstrates that the peptide is the leucinamide form (peptide 111–122, Fig. 1), as was anticipated by the presence of a Gly-Arg-Arg sequence following the leucine residue (Fig. 7). Because the radioimmunoassay is approximately 1000 times more sensitive for the detection of the amidated compared to carboxyiated peptide, the latter peptide may also be present but undetectable.

*Immunocytochemical Colocalization of Glucagon-related Peptides in Pancreatic and Intestinal Cells*—By light microscopy antisera to glucagon, GLP-I (both full-length peptide of 37 amino acids and the peptide of 17 amino acids *overlined* in Fig. 1), GLP-II and intervening peptide II amide all produced bright immunofluorescence on consecutive thin sections of




D.

FIG. 10. **Northern analyses of colonic and pancreatic poly(A⁺) RNA hybridized with individual $^{32}$P-labeled oligonucleotides, densitometric scans of the autoradiograms, and locations of individual oligonucleotide-hybridization probes in the sequences of pancreatic preproglucagon mRNA and glucagon gene.** Poly(A⁺) mRNA was prepared from colon and pancreas, transferred to nylon membrane, and hybridized to individual $^{32}$P-labeled oligonucleotides (E1–E6) as described under "Experimental Procedures." Autoradiograms (*line A*) were scanned densitometrically. Individual densitometric tracings are shown below each autoradiogram (*line B*). Note that two separate strips of membrane each were separately hybridized consecutively with three of the six oligonucleotides as indicated above each strip. In the glucagon gene diagram (*line C*), *lines* represent introns (*IA–IE*) and *boxes* exons (*E1–E6*). In the mRNA diagram (*line D*) *un-tx* indicates untranslated regions. The translated region contains from 5′ toward 3′ ends the signal peptide (*S, stippled box*), N-terminal extension (*N = cross-hatched boxes*), glucagon (*Gluc = solid box*), intervening peptide I (*IP1 = cross-hatched box*), glucagon-like peptide I (*GLP-1 = solid box*), intervening peptide II (*IP-II = cross-hatched box*) and glucagon-like peptide II (*GLP-II = solid box*). Amino acids at the junctions of the various peptide domains of preproglucagon are indicated by *letters* above the mRNA diagram. The sequences of the oligonucleotide hybridization probes and their locations on preproglucagon mRNA are: exon 1 probe, ~TTCCTCGAGGTGGACAGATGTGGA (bases 2–25); exon 2 probe, TACTTTCTGGCAAATGTAGCACC (bases 61–82); exon 3 probe, CTAAAACACGTCACCAACTAC (bases 277–297); exon 4 probe, TTCCTTAAGTAACGAACCGACCA (bases 409–431); exon 5 probe, AAGAGAC-TACTCTACTTGTG (bases 511–531); exon 6 probe, GTTGTAGAGGACGGTCAGTGAACCC (bases 633–657). The base numbers given in parentheses correspond to the numbering of the bases of preproglucagon mRNA (Heinrich *et al.*, 1984a). In the schematic representation of the glucagon gene, the location of each oligonucleotide hybridization probe that corresponds to the densitometric tracing and autoradiogram above it are indicated by *wavy lines*.

pancreatic islet A cells located in the periphery of the islets (Fig. 8*A*). Similar immunofluorescence was detected in the population of endocrine cells in the intestinal mucosa (L cells) with antisera against glicentin, GLP-I (1–37), GLP-II, and intervening peptide II amide (Fig. 8*B*). Therefore, it appears that all the peptide-encoding exons of the preproglucagon gene are expressed in the identical cells of the islets and intestines.

By immunoelectronmicroscopy, using the protein A-gold technique, the antisera to GLP-I, GLP-II, and intervening peptide II amide specifically labeled the secretory granules of the rat pancreatic A-cells (data not shown). On human islets, only the GLP-1 antiserum prepared to the 37-amino acid peptide produced an intense labeling on the A-cell secretory granules (Fig. 9). The labeling was located over the dense, internal core rather than the halo of the granule. A similar distribution of labeling of the human A-cell was demonstrated previously for glucagon using a C-terminally directed antiglucagon serum (Ravazzola and Orci, 1980). A quantitative evaluation of the number of GLP-I-specific gold particles located on the $\alpha$-granules (300 granules evaluated) showed that 94% of the particles were located over the dense core. We had shown previously that glucagon and glicentin are topologically segregated in the $\alpha$-granules of human A-cells; glicentin-specific particles are located in the halo rather than the core of the granules (Ravazzola and Orci, 1980).

*Northern RNA Analysis*—Inasmuch as only three exons of the preproglucagon gene are required to encode glicentin, the remaining three exons could differ in sequence from the pancreatic exons. To investigate this possibility, we synthesized six oligonucleotides each complementary to a separate exon of the rat glucagon gene. Hybridization of parallel blots of intestinal and pancreatic RNA individually with each of the six oligonucleotides showed that all six complementary oligonucleotides hybridize with the same intestinal mRNA that is identical in size with the pancreatic preproglucagon mRNA (Fig. 10, *line A*). Because of the possibility that two mRNAs may differ in sequence but not in length, *i.e.*, may be superimposed in the autoradiogram, we also determined the ratio of the densities of the autoradiographic images resulting from hybridization of the pancreatic and intestinal mRNAs with each of the six oligonucleotides. The ratio was the same for all pairs of mRNAs compared, and for all oligonucleotides hybridized (Fig. 10, *line B*); the ratio was 1.7 for strips E-1, E-5, and E-6 and 1.5 for strips E-2, E-3, and E-4.

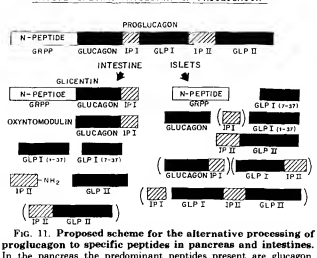



FIG. 11. **Proposed scheme for the alternative processing of proglucagon to specific peptides in pancreas and intestines.** In the pancreas the predominant peptides present are glucagon, glucagon-like peptide I (*GLP-I*), and a large form of glucagon-like peptide II (*GLP-II*). In the intestine the major peptides are glicentin, oxyntomodulin, GLP-I, GLP-II, and intervening peptide II-amide (*IP-II-NH*$_2$). Other, less well characterized peptides that are likely to be present in the tissues are bracketed by *parentheses*. *GRPP* indicates glicentin-related polypeptide which is presumed to be present in pancreas based on earlier studies (Baldissera and Holst, 1984).

## DISCUSSION

The results of these studies demonstrate that glucagon, the two glucagon-like peptides, and an amidated intervening peptide are differentially cleaved from proglucagon in pancreas and intestine. Further, specific antigenic epitopes for each of the peptides are present in the identical cells of the pancreas and intestine. Our observations also indicate that only a single mRNA is present in intestine and that it is similar, if not identical in sequence, with the pancreatic preproglucagon mRNA. In conjunction with previous evidence indicating the existence of a unique glucagon gene in the rat genome (Heinrich et al., 1984b), our data support the contention that glicentin and glucagon are derived from the same polyprotein precursor encoded by the same mRNA without detectable exonic rearrangements.

The specific patterns of processing of proglucagon in pancreas and intestine are somewhat more complex than was initially anticipated (Fig. 11). Although the efficient formation of glucagon in the pancreas, and glicentin and oxyntomodulin in the intestine, confirm existing information derived from glucagon immunoanalyses of the tissues, we discovered the liberation of at least three forms of GLP-I in the pancreas and intestine as well as the intestinal-specific liberation of GLP-II and the amidated form of intervening peptide II.

Our findings of both large and small forms of GLP-I in pancreas are consistent with the results reported by Patzelt and Schiltz (1984) and by George *et al.* (1985). However, we found that much of the GLP-I immunoreactivity is in the form of the peptides of 37 and 31 amino acids, an observation that differs somewhat from the earlier studies in which the major pancreatic peptide detected consists of a large form of GLP-I presumably covalently linked to IP-II and GLP-II (Patzelt and Schiltz, 1984; Uttenthal *et al.*, 1985). It is likely, however, that our antisera which effectively recognizes the GLP-I peptide does not equally recognize the immunodeterminant of GLP-I when in the form of a precursor, whereas the antisera used by these other workers does. We found a considerable difference in the relative amounts of large and small forms of GLP-I when two different antisera were used in the radioimmunoassay (Fig. 3). On the other hand, the GLP-I used by Uttenthal *et al.* (1985) for immunization was GLPI (1-19); GLPI 1-37 and GLPI 7-37 may not be readily detected by such an antisera (Fig. 4). Thus, it remains a possibility that differences in sensitivities of antisera prepared to the different GLP-I peptides accounts for the apparent differences in amounts of large and small forms of GLP-I in the two studies. We detected only a large form of GLP-II in the pancreas, and also in the intestine a larger GLP-II was present at a level of about 50% of the amounts of GLP-II peptide. Our interpretation of the structure of these large forms of GLP-II is that they consist of GLP-II linked to IP-II in the intestine and possibly also to GLP-I in the pancreas.

The finding of a GLP-I (7-37) peptide is important because of the unusual configuration of the sequence of the GLP-I (1-37). The homology of the GLP-I sequence with the corresponding sequences in the two anglerfish preproglucagons (Lund *et al.*, 1983) and with the other members of the glucagon supra-gene family of peptides, begins with the histidine at position 7 preceded by an arginine at position 6. It appears that the GLP-I (1-37) may be a "prohormone" or precursor for the GLP-I (7-37) peptide that is processed from GLP-I (1-37) by cleavage at the single arginine residue at position 6.

The liberation of GLP-I in the pancreatic islets raises the possibility that it may regulate endocrine or paracrine secretion of other islet hormones. We[2] and others (Schmidt *et al.*, 1985) have observed an insulinotropic effect of GLP-I on islet cells *in vitro*. Likewise, the liberation of both glucagon-like peptides I and II, as well as the amidated intervening peptide, in the intestines suggests a biological role of these peptides in the intestinal tract. The finding that the intervening peptide is amidated implies that it is likely to be a bioactive peptide (Tatemoto and Mutt, 1980). It is possible that the glucagon-like peptides are the intestinal growth-promoting factors detected in extracts of endocrine tumors producing enteroglucagon (Gleeson *et al.*, 1971) and may also be the factors responsible for stimulation of intestinal crypt cell production Uttenthal *et al.*, 1982). Recently it has been reported that synthetic human glucagon-like peptides I and II activate adenylate cyclase in brain membranes (Hoosein and Gurd, 1984) and apparently have no actions on liver (Hoosien and Gurd, 1984; Ghiglione *et al.*, 1984).

The demonstration that glicentin and glucagon appear to be derived from the same polyprotein precursor implicates tissue-specific differences in the post-translational processing of pre-proglucagon as the source of the complex differences in peptide patterns found between gut and pancreas. These findings provide an impetus for investigations of the molecular mechanisms that are responsible for the tissue-specific processing of preproglucagon.

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